PROOPIOMELANOCORTIN EXPRESSION AND PROCESSING IN THE OVINE PARS INTERMEDIA

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Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree.

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Abstract

Proopiomelanocortin expression and processing in the ovine pars intermedia

In the sheep, peripheral blood plasma concentrations of alpha melanocyte stimulating hormone (αMSH) undergo marked seasonal fluctuations and are maximal in late summer and autumn. The pituitary pars intermedia is believed to be the principle source of this circulating αMSH which is synthesised from the proopiomelanocortin (POMC) precursor through the proteolytic action of the prohormone convertases, PC1 and PC2. This thesis describes studies into the effect of season and photoperiod on the αMSH-synthetic activity of the pars intermedia in the highly photoperiodically-responsive Soay sheep. The influence of the hypothalamus and dopaminergic inhibition was also studied. The localisation and expression of POMC mRNA, POMC-derived peptides and both PC1 and PC2 mRNAs and proteins were measured by in situ hybridisation, RNase protection assay and immunocytochemistry; specific riboprobes were developed for the project. The presence of the cellular proliferation marker, PCNA, was used as an index of cellular proliferation.

In the first study, the general anatomy of the ovine pituitary gland was investigated using the new methodology. POMC mRNA was highly expressed in melanotrophs in the pars intermedia and in the corticotrophs dispersed in the pars distalis. The POMC-derived peptides, ACTH and αMSH both occurred in the pars intermedia while in the pars distalis ACTH predominated while αMSH was only weakly expressed. PC1 mRNA and protein was also expressed both in the pars distalis and the pars intermedia with a distribution similar to that of POMC, as well as in the pars nervosa. PC2 mRNA was expressed in the pars intermedia while PC2 protein occurred principally in the pars intermedia and pars nervosa. Like αMSH, PC2 protein was expressed weakly in the pars distalis. The second study investigated the changing activity of the pars intermedia at four seasonal time points in Soay ewes living outdoors and exposed to natural photoperiod (n=5/group). Expression of POMC and PC1 mRNA was significantly greater in the summer and autumn compared to the winter and spring. The autumnal increase in pars intermedia activity was also characterised by an increase in cellular proliferative activity and hypertrophy of melanotrophs. The effect of photoperiod on the activity of the pars intermedia was investigated in the third

study in which Soay rams were exposed to an artificial lighting regimen of alternating 16-weekly periods of long days (LD, 16L 8D) and short days (SD, 8L 16D) designed to entrain the seasonal αMSH cycle and were killed following treatment under LD and SD (n=4-5/group). Expression of POMC, PC1 and PC2 mRNA was enhanced in the SD group although this was statistically significant only for PC1 due to large individual variation.

The fourth study investigated the role of the hypothalamus in the photoperiodic regulation of the pars intermedia in control and long term hypothalamo-pituitary disconnected (HPD) Soay rams (n=8/group) exposed to an artificial lighting regimen (as above) for 80 weeks, a subset of which were later killed under long days (n=3/group). In control rams, circulating αMSH concentrations varied markedly with photoperiod with a 10-fold increase from the minimum under LD to the maximum under SD. In HPD rams αMSH concentrations increased following surgery (significantly above control values) and then declined in the longer term; there were no cyclical changes associated with the switches in photoperiod. The expression of POMC, PC1 and PC2 mRNA in the pars intermedia tended to be reduced in the long term HPD rams compared with controls exposed to short days, although this was statistically significant only for PC1. The pars intermedia was enlarged in size in HPD rams.

In the final study, the potential importance of dopamine (DA) as an inhibitory regulator of the pars intermedia was investigated in Soay rams living outdoors under natural photoperiod. Animals were treated chronically with a long term DA agonist (parlodel LA, 16.7mg/animal iv every 5-7 days for 40 days) in July and August at the time of the seasonal maximum in αMSH secretion. Untreated rams were used as controls (n=3/group). Treatment with the DA agonist resulted in sustained (70%) reduction in circulating concentrations of αMSH. This was associated with a significant reduction in the expression of POMC, PC1 and PC2 mRNA in the pars intermedia and a decrease in cell proliferation and cell size.

Overall, the results support the conclusion that the seasonal cycle in blood plasma concentrations of αMSH in sheep are mediated by the photoperiodic co-regulation of POMC, PC1 and PC2 gene expression which dictates the rate of synthesis of αMSH by the pars intermedia. This photoperiodic regulation is transduced through direct hypothalamic innervation of which dopaminergic inhibitory control is likely to predominate. Thus, the ovine pars intermedia is a seasonally dynamic tissue with potentially important roles established previously in the regulation of carbohydrate metabolism, immunomodulation and pigmentation.

Abbreviations

AE	Autumn equinox
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APAAP	Alkaline phosphatase anti alkaline phosphatase
BSA	Bovine serum albumin
CL	Corpus luteum
CLIP	Corticotropin-like intermediate lobe peptide
CPE	Carboxypeptidase E
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
DTT	Dithiotheitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N ,N ,N',N'-tetraacetic acid
GABA	γ-aminobutyric acid
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D thiogalactopyranoside
LHβ	Luteinising hormone beta-subunit
MOPS	(N-Morpholino) propanesulphonic acid
NBT	nitrobluetetrazolium salt
PAL	Peptidylhydroxyglycine-a-amidating lyase
PAM	Peptidyl glycine alpha-amidating monooxygenase
PCR	Polymerase chain reaction
PC1	Prohormone convertase 1
PC2	Prohormone convertase 2
PCNA	Proliferating cell nuclear antigen
POMC	Proopiomelanocortin
RNA	Ribonucleic acid

RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SE	Spring equinox
SEM	Standard error of the mean
SS	Summer solstice
Taq	Thermus aquaticus
TBS	Tris-buffered saline
WS	Winter solstice

Chapter 1

General Literature review

1.1 Introduction

The ability to respond to changes in the external environment is one of the fundamental requirements of all living organisms. Mammals living in cold and temperate climates experience substantial and predictable variations in environmental conditions such as temperature and food availability and show marked adaptive seasonal cycles in reproduction, growth and pelage. The endocrine control of these adaptive characteristics is under physiological regulation by the hypothalamus, which receives, integrates and conveys information about external factors to the pituitary gland. The pituitary gland amplifies these hypothalamic responses and generates conspicuous circulating humoral signals which temporally co-ordinate the peripheral physiological systems. Thus the ability of the hypothalamo-pituitary axis to respond to environmental cues is central in physiological and behavioural adaptations to seasonal climates.

The sheep is a well studied example of a mammal adapted to living in such seasonal environments. Seasonal changes in food availability, with abundant food in summer and food shortages in winter exert considerable selective pressure to accumulate fat reserves before the winter and to restrict the mating season to the autumn. The hypothalamo-pituitary axis is central in the physiological systems controlling such seasonal adaptations. There are pronounced seasonal cycles in the secretion of hormones from the pituitary gland such as luteinising hormone (LH), follicle-stimulating hormone (FSH) and prolactin and these are involved in the regulation of reproduction and pelage growth (Lincoln and Short, 1980). While there has been extensive research conducted to understand the seasonal regulation of the reproductive axis, there is a rather smaller body of literature on the physiological regulation of the seasonal cycle in growth and metabolism (Bronson, 1989). Conspicuous seasonal cycles in circulating alpha-melanocyte-stimulating hormone (αMSH) and beta-endorphin (βEND) are observed in the sheep which correlate closely with the seasonal changes in voluntary food intake and body weight (Ebling and Lincoln, 1987). Both of these peptides are co-secreted from the pituitary gland and are derived from a single precursor, proopiomelanocortin (POMC). The POMC protein is cleaved into smaller peptides by the endoproteolytic action of the prohormone convertases PC1 and PC2 and these

peptides are further modified by other processing enzymes before being secreted. The post-translational processing of POMC is tissue specific and results in the biosynthesis of a combination of several different peptides. Thus the bioactivity of the products of the POMC precursor are governed by the processing activity of a particular tissue. The cellular events governing the processing of POMC are therefore as important as POMC gene expression itself in generating bioactive peptides such as αMSH and βEND. In the sheep it is believed that most circulating αMSH is derived from the pituitary pars intermedia (Engler *et al.*, 1989). This thesis aims to investigate the seasonal regulation of POMC gene expression and the processing of the POMC precursor protein in the pars intermedia of the highly photoperiodically responsive Soay sheep.

To provide an introduction to the subject of this thesis in the next section the literature concerning the anatomy of the pars intermedia, the precise nature of the regulation of its activity and the synthesis of its principle products will be reviewed.

1.2 Neuroendocrine control of pars intermedia POMC expression and secretion

1.2.1 General anatomy of the ovine pituitary gland

The pituitary gland is the principle source of POMC-derived peptides present in the peripheral blood (Autelitano *et al.*, 1989; Lundblad and Roberts, 1988) and expresses POMC mRNA at a level 1000-fold greater than the hypothalamus (Smith and Funder, 1988). All vertebrate species possess a pituitary gland (Wingstrand, 1966) and within the mammals the pituitary gland is composed of a consistent number of tissues of different developmental origins. These tissues, of which the pars intermedia is one, have distinct structural and functional properties in the adult pituitary gland (Figure 1.1). The pituitary gland is developmentally derived in part from the brain and in part from the oral fossa (Figure 1.2). The region derived from the oral fossa develops into the pars intermedia and anterior lobe. The anterior lobe, sometimes called the glandular lobe, has an endocrinologically active and heterologous cell population and is divided functionally and histologically into the pars tuberalis, the zona tuberalis and the pars distalis. The region derived from the brain forms the remaining part of the posterior lobe comprised of the pars nervosa and median eminence (Atwell, 1918). The grouping of the pars intermedia into the posterior lobe reflects its close physical association with the pars nervosa but does not reflect its developmental origin.

1.2.2 Development of the ovine pituitary gland

The anterior and posterior lobes of the pituitary gland are formed by the folding and migration of two distinct tissue layers derived from the brain and the oral fossa. The part derived from the oral fossa is initially formed from a shallow infolding anterior to the oral plate towards the brain and this gives rise to Rathke's pouch (Figure 1.2). Rathke's pouch becomes more closely associated with the neural lobe and the remaining connection to the oral cavity, the hypophyseal stalk, becomes more constricted and is eventually lost (Atwell, 1918). In the sheep this occurs by day 31 of gestation (Perry *et al.*, 1982). The developmental fate of the cells forming Rathke's pouch is probably already determined before the pouch is formed since homeobox genes associated with the structure are expressed long before it forms (Treier and Rosenfeld, 1996). The distinct distribution of particular types of secretory cells of the pars distalis is likely to result from a complex transcriptional-cascade in development, where developmental fate tends to be influenced by relative position (Treier and Rosenfeld, 1996), although there are no clear boundaries between functional cell types in the pars distalis. Factors released from the floor of the primitive diencephalon are also thought to influence pituitary cell differentiation (Lamonerie *et al.*, 1996).

The part of the developing pituitary gland derived from the brain forms the pars nervosa which remains attached to the brain. This also is derived from a pouch-like structure, an outgrowth of the third ventricle, but the lumen of the pars nervosa is obliterated by numerous foldings early in pituitary gland development (Atwell, 1918). Functionally the pars nervosa is an outgrowth of the hypothalamus, containing numerous nerve fibres and terminals from neurons originating in the hypothalamus. Rathke's pouch pushes up against the neural lobe which projects down ventrally. The position of the neural lobe changes throughout pituitary gland development so that it projects posteriorly and lies flat underneath the brain wall in the adult gland (Atwell, 1918). The tissue forming Rathke's pouch develops into the pars intermedia at its neural aspect and into the anterior lobe at the anterior aspect. The lumen of this pouch separates the anterior lobe from the pars intermedia and through embryonic development undergoes a degree of regression but survives as the hypophyseal cleft or hypophyseal vesicles in the adult gland. Ultrastructural analysis suggests that the residual cleft is empty in the ovine fetal pituitary but in the adult contains a colloid like material acquired soon after birth (Perry *et al.*, 1982). In the sheep the posterior aspect of Rathke's pouch, which gives rise to the pars intermedia, is still undifferentiated at day 26 of gestation. By day 40, only the cells lining the residual cleft, which are not fully differentiated melanotrophs, show any sign of differentiation, and differentiation of the rest of the tissue is not observed until day 50. While corticotrophs are present

at day 95 of gestation (Challis and Brooks, 1989), the fully functional melanotroph is not observed until day 100 (Perry *et al.*, 1982). Follicles, common in adult pars intermedia, are not seen until 120 days of gestation (Perry *et al.*, 1982). The pars intermedia of the new-born lamb is approximately ten cells deep and increases to about 15 cells in the adult. Furthermore the perinatal pars intermedia is more richly vascularised than in the adult and these blood vessels occur from gestational day 100 onwards (Perry *et al.*, 1982).

There are reports suggesting that the corticotroph population changes morphologically through the development of the ovine pars distalis. The predominant fetal corticotroph phenotype is large and columnar while the adult phenotype is stellate and darker. It may be that the processing of the POMC molecule is less extensive in the fetal type so explaining the incidence of high molecular weight POMC peptides in fetal circulation which appear to antagonise the effects of ACTH on the adrenal cortex. The gradual change from fetal to adult phenotype can be reversed by adrenalectomy, suggesting that glucocorticoids promote this change (Challis and Brooks, 1989). The distribution of ACTH-immunoreactive corticotrophs also tends to shift from the superior part of the pars distalis, adjacent to the pars tuberalis, to the inferior pars distalis from day 60 to parturition (Matthews *et al.*, 1994).

The pars tuberalis, the most anterior and dorsal part of the anterior lobe is thought to be derived from lateral outgrowths of Rathke's pouch which migrate up to and from processes that surround and fuse enclosing the median eminence (see Figure 1.2) (Atwell, 1918). This part of the anterior lobe is functionally and histologically distinct from the pars distalis.

1.2.2.1 Blood supply to the pituitary gland.

The anterior and posterior lobes of the pituitary gland are vascularised from two sources, both of which ultimately arise from the internal carotid artery. The anterior lobe is richly vascularised by capillaries arising from the anterior hypophyseal artery as well as from the portal vessels which run through the median eminence and into the zona tuberalis (Green and Harris, 1947; Harris, 1947). The blood supply from the anterior hypophyseal artery first runs through the pars tuberalis before joining the portal vessels (Fitzgerald, 1979) which are observed from gestational day 45 in the sheep (Challis and Brooks, 1989).

The posterior lobe is vascularised by the posterior hypophyseal artery and thus it is unlikely that the posterior lobe receives any portal blood from the median eminence (Harris, 1947). The rat pars intermedia is reported to be poorly

vascularised but the literature suggests that this is less the case in the sheep (Perry *et al.*, 1981).

1.2.2.2 The development of the pars intermedia innervation

The activity of the pars intermedia is believed to be regulated by direct hypothalamic innervation from the hypothalamus. In development, regulatory neurons migrate towards the pars intermedia from the hypothalamus. In the adult gland there is a very close association between the pars intermedia and pars nervosa which becomes apparent if attempts are made to separate the two tissues. In the developing rabbit fetus, pronounced projections invading the pars intermedia from the pars nervosa have long been reported (Atwell, 1918). In the sheep, anterograde tracers show that tyrosine hydroxylase (the rate limiting enzyme in dopamine synthesis) -expressing neurons originate at least in part from the A15 nucleus in the retrochiasmatic area of the hypothalamus. Dopaminergic neurons from the A15 also project nerve fibres into the median eminence and pars nervosa (Gayrard *et al.*, 1995). In the rat, αMSH promotes the outgrowth and expression of tyrosine hydroxylase in cultured fetal neurons, suggesting that αMSH itself acts as a neurotrophic factor, promoting differentiation of the dopaminergic neurons and innervation of the melanotrophs (Egles *et al.*, 1998). The development of this innervation extends into the perinatal period in the rat (Chronwall *et al.*, 1998).

In the sheep, the innervation of the pars intermedia is already partially developed by 125 days of gestation since the pars intermedia is capable of responding to stress induced as a result of hypoxemia by decreased POMC mRNA expression. The response to hypoxemia by the pars intermedia becomes more pronounced in older fetuses (Braems *et al.*, 1996) implying that development of the system regulating the activity of the pars intermedia is still ongoing at this stage. D2 dopamine receptors are expressed by day 130 of gestation in the fetal sheep pars intermedia since POMC mRNA is reduced to half pretreatment levels by chronic treatment with the dopamine agonist, bromocriptine (Matthews *et al.*, 1996).

1.2.3 Anatomy of the adult pituitary gland

1.2.3.1 The pars intermedia

The ovine pars intermedia is a lobular tissue with a cell population predominantly composed of polyhedral melanotrophs with uniform ovoid nuclei. Compared to the pars distalis, the pars intermedia is relatively poorly vascularised and is often separated from the pars distalis by a prominent hypophyseal cleft, although in

places the pars intermedia and pars distalis fuse with no clear boundary between the two tissues (Perry *et al.*, 1981). The boundary between the pars intermedia and pars tuberalis is also rather poorly defined (Dawson, 1937). In some species the pars intermedia is reported to enclose the pars nervosa, particularly close to the infundibular stalk (Atwell, 1918; Dawson, 1937). In the sheep the pars intermedia forms a layer of melanotrophs approximately 15-20 cells thick. Melanotrophs are rich in granular endoplasmic reticulum and secretory granules, reflecting their endocrine function, although the number of granules varies considerably from cell to cell (Perry *et al.*, 1981). A significant degree of cell proliferation in the pars intermedia is a normal feature of development and may also occur in the adult sheep since pars intermedia hypertrophy is observed after removal of hypothalamic influence (Clarke *et al.*, 1983) and cell proliferation has been observed in the adult rat pars intermedia (Horiuchi *et al.*, 1994). In the developing ovine pars intermedia, mitosis has been observed in apparently endocrinologically active melanotrophs containing secretory granules at 100 gestational days (Perry *et al.*, 1982).

In addition to the melanotroph, four additional cell types occur in the ovine pars intermedia. These are described as pars distalis-like glandular cells, interstitial cells, follicular cells and cleft lining cells (Perry *et al.*, 1981). The pars distalis-like cells, representing less than 5% of total pars intermedia cells, were characterised using electron microscopy rather than by any endocrine or functional aspect. There are, however, immunocytochemical reports of a few gonadotrophs invading the pars intermedia from the pars distalis in the mouse and rat (Stoeckel and Porte, 1984). The interstitial cells of the pars intermedia are characterised by elongated indented nuclei and the absence of granular endoplasmic reticulum, suggesting a non secretory role for these cells.

Follicles sometimes containing acellular colloid-like material are often observed in the ovine pars intermedia, usually close to the margin with the pars nervosa. These are lined by follicular cells which ultrastructurally resemble interstitial cells and together with them represent less than 5% of the cells of the pars intermedia (Perry *et al.*, 1981).

The hypophyseal cleft is lined by a mixed cell population on the pars intermedia side, mainly triangular cells, tapered basally and bearing microvilli extending into the cleft. Another epithelial cell type is commonly observed which occurs in clusters and is ciliated at the cleft aspect (Perry *et al.*, 1981).

1.2.3.2 The pars nervosa

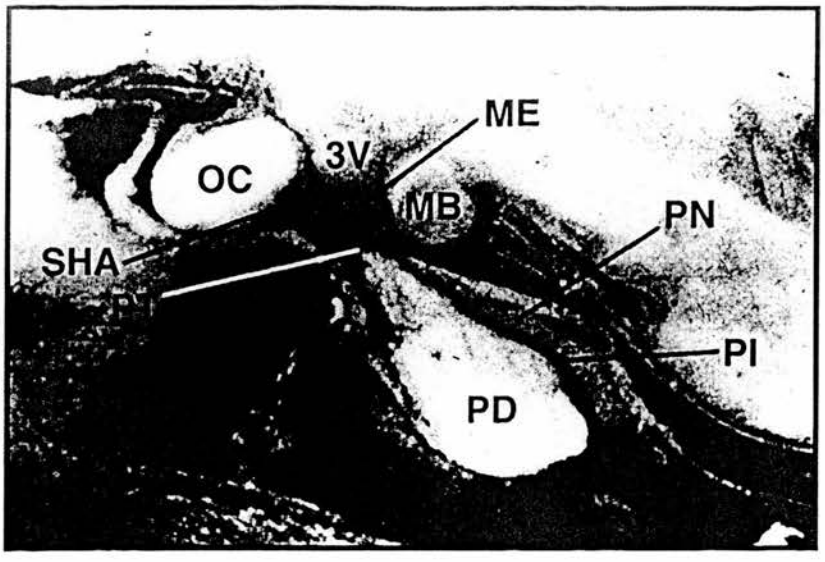
The pars nervosa is made up of neuronal axons which project into this tissue from the hypothalamus. The principle types of neurons are those which produce vasopressin and those which produce oxytocin. In addition a newly discovered neuropeptide, secretoneurin, is synthesised by the bovine pars nervosa and, like POMC, is produced from a larger precursor, secretogranin II (Egger *et al.*, 1994). The pars nervosa also contains a number of neurons whose function is to regulate the activity of the pars intermedia. These are principally dopaminergic, GABAergic and β-adrenergic (Chronwall *et al.*, 1998).

1.2.3.3 The median eminence

The median eminence is also structurally a part of the hypothalamus containing many neuroendocrine synapses which secrete hypothalamic peptides into a rich vascular system which supplies the pars distalis. Essentially, the median eminence is formed from an outfolding of the post-optic hypothalamus along the route of the portal blood system (Wingstrand, 1966) It is enclosed by the pars tuberalis and contains the axons of neurons projecting from the hypothalamus into the pars nervosa. Unusually for a central tissue, the median eminence lacks a blood-brain barrier (Garcia de Yebenes *et al.*, 1997), suggesting that it may be possible for pituitary factors to influence this tissue.

1.2.3.4 The pars tuberalis

The pars tuberalis is present in all mammals with a few exceptions, and lies at the most anterio-dorsal part of the pituitary gland within the pia mater of the brain, posterior to the optic chiasm (Atwell, 1918). In the rat, the pars tuberalis may be the first part of the pituitary gland to become functionally active (Fitzgerald, 1979). The pars tuberalis forms a collar several cells deep around the median eminence. These cells are of a mixed secretory and non-secretory population and are thought to include some endocrine cells usually associated with the pars distalis, particularly migrating gonadotrophs which are most abundant towards the pars distalis (Fitzgerald, 1979). These gonadotrophs become more abundant in the rat after removal of the rest of the pituitary gland (Stoeckel and Porte, 1984). The pars tuberalis is the most richly vascularised part of the pituitary gland (Fitzgerald, 1979). Cerebrospinal fluid is thought to be able to gain access to the cells of the pars tuberalis through cellular cords distributed widely through the tissue (Stoeckel and Porte, 1984). Abundant melatonin receptors are expressed in the pars tuberalis and zona tuberalis, but are absent elsewhere in the ovine pituitary gland. These are involved in the seasonal control of prolactin secretion in the sheep since this is the only pituitary hormone that responds to changes in photoperiod when the pituitary



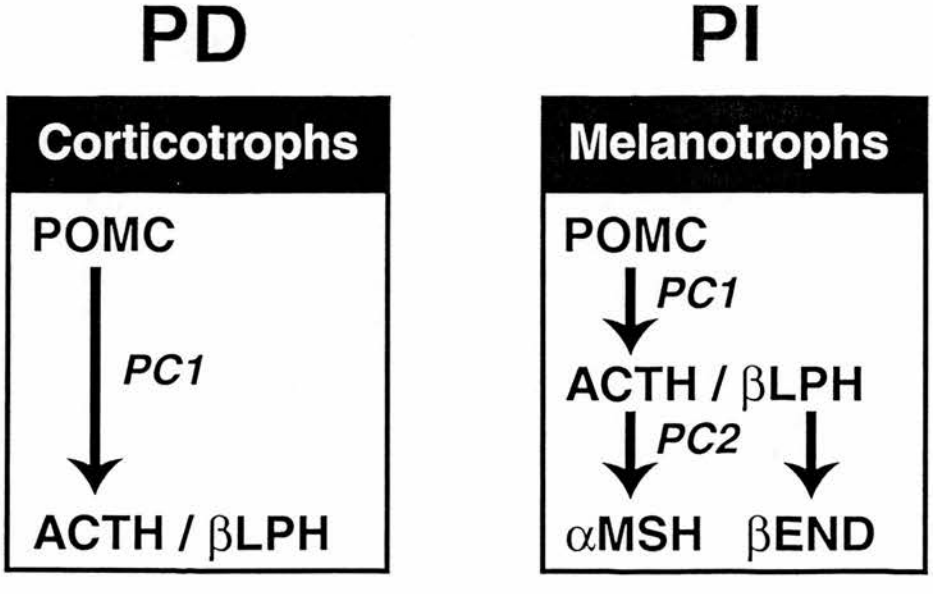


Figure 1.1. Photograph of the ovine pituitary gland and hypothalamus in sagittal plane (top) and schematic diagram showing the differential processing of POMC in the two POMC-expressing pituitary cell types (bottom). MB- mediobasal hypothalamus, ME- median eminence, OC- optic chiasma, PD- pars distalis, PI- pars intermedia, PN- pars nervosa, PT- pars tuberalis, 3V- third ventricle. Adapted from Clarke et al. (1983) Neuroendocrinology 36: 376.

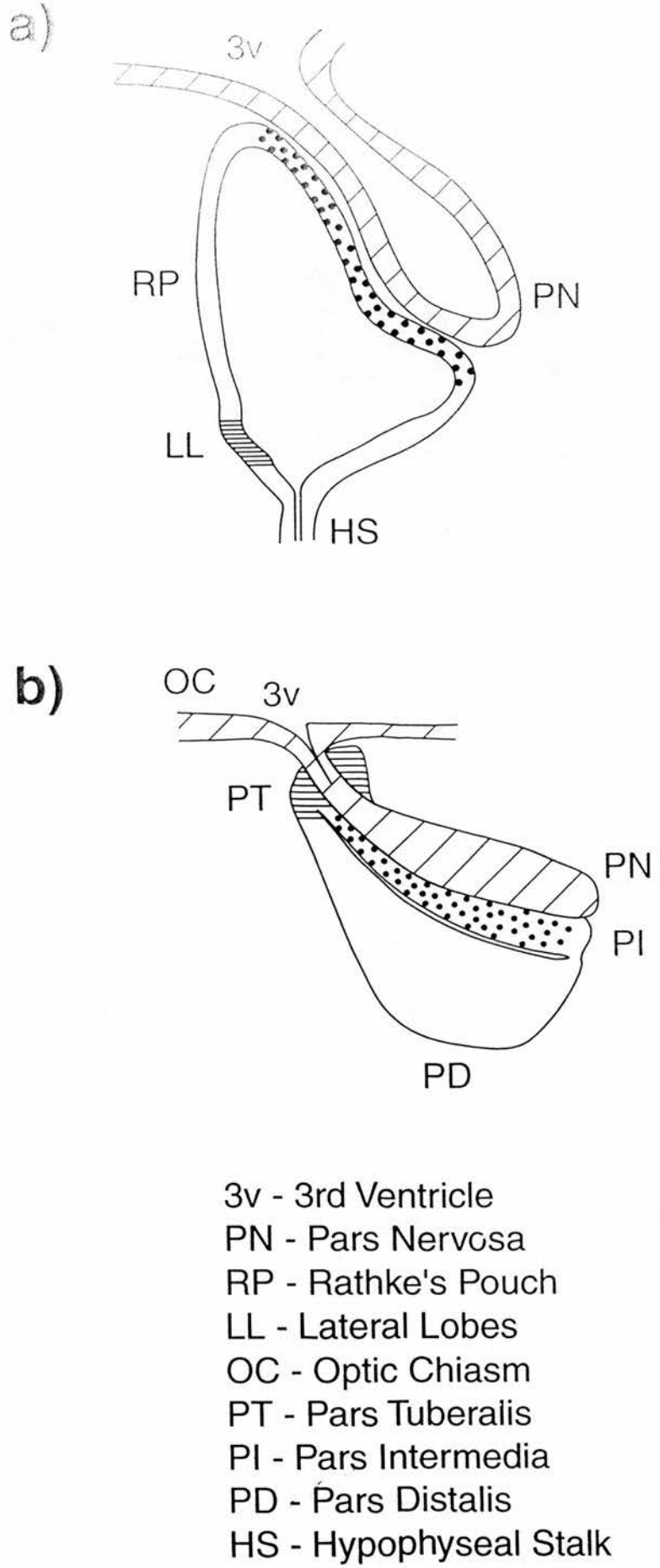


Figure 1.2. General anatomy of the developing (a) and adult (b) mammalian pituitary gland, both in sagittal section.

is disconnected from the hypothalamus (Lincoln and Clarke, 1995). Reported photoperiodic and seasonal changes in secretory cell morphology in species other than the sheep further implicate the pars tuberalis as a modulator of pars distalis function (Rutten *et al.*, 1988; Wittkowski *et al.*, 1984).

1.2.3.5 The zona tuberalis

The zona tuberalis was first identified and considered as separate from the pars distalis on the basis of a lack of eosinophillic cells (Dawson, 1937). The zona tuberalis lies at the anterior pole of the pituitary gland and seems to form an extension of the pars tuberalis adjoining the pars distalis. There is no clear boundary between the zona tuberalis and the pars tuberalis or pars distalis (Dawson, 1937). This tissue contains many blood vessels and sinusoids (Harris, 1947) and is reported to contain a large proportion of gonadotrophs in the sheep (Skinner and Robinson, 1995).

1.2.3.6 The pars distalis

The pars distalis contains the majority of endocrinologically active cells in the ovine pituitary, including ACTH-secreting corticotrophs, prolactin-secreting lactotrophs, LH and FSH-secreting gonadotrophs, growth hormone-secreting somatotrophs and thyroid-stimulating hormone-secreting thyrotrophs. Immunocytochemical studies of these cell types has revealed that each show a unique pattern of distribution and some of these cell types are also present in the zona tuberalis (Girod *et al.*, 1982; Girod *et al.*, 1983; Girod *et al.*, 1985; Girod *et al.*, 1986; Sasaki *et al.*, 1992; Skinner and Robinson, 1995). Follicular-stellate cells, whose function is not clear, also occur extensively in the pars distalis (Jabbour *et al.*, 1997; Mendez *et al.*, 1998).

1.2.4 Regulation of POMC-derived peptide expression and secretion

1.2.4.1 Pituitary gland POMC expression

Within the pituitary gland, POMC is expressed both by the pars distalis of the anterior lobe and the pars intermedia of the posterior lobe. It is generally accepted that the pars intermedia is the source of most of the peripheral circulating βEND and αMSH in the sheep and this is substantiated by several lines of evidence. First, in the seasonal cycle in plasma αMSH and βEND concentration in sheep, αMSH concentrations correlate closely with βEND levels with a molar ratio between the two hormones close to 1:1 (Lincoln and Baker, 1995). This suggests that these

two POMC-derived products are co-secreted from the same tissue and are subject to the same regulatory mechanism.

Secondly, ACTH, which is an intermediate in the processing of αMSH from the precursor, POMC, is also secreted into the circulation from the pituitary gland. It is thought that the corticotrophs of the pars distalis secrete most of this circulating ACTH in response to hypothalamically-derived humoral releasing factors (Smith and Funder, 1988). Plasma ACTH correlates poorly with photoperiod and does not correlate well with αMSH in the long term, suggesting that the observed changes in αMSH and βEND are due to the secretory activity of a cell type other than the corticotroph, specifically the pars intermedia melanotroph (Lincoln and Baker, 1995). Moreover, circulating cortisol, which is secreted in response to ACTH, is also not clearly affected by photoperiod (Brunet and Sebastian, 1991; Ebling and Lincoln, 1987). Ultradian fluctuations in ACTH with a frequency of minutes, correlate only partially with fluctuations in αMSH secretion in the sheep (Engler *et al.*, 1989; Hagan and Brooks, 1996). Such secretion of ACTH with αMSH has also been demonstrated in ovine fetal melanotrophs in vitro (Fora *et al.*, 1996). Since ACTH is also produced by the pars intermedia as an intermediate in αMSH biosynthesis, the partial correlation of such short term fluctuations of αMSH to ACTH is consistent with the hypothesis that the pars distalis is the source of most circulating ACTH while the pars intermedia secretes most circulating αMSH with a small quantity of ACTH.

Thirdly, glucocorticoid receptors are expressed by corticotrophs but are not usually expressed in the pars intermedia (Autelitano *et al.*, 1989). Dexamethasone, a synthetic glucocorticoid receptor agonist, inhibits the secretion of ACTH, but not αMSH, in the sheep (Engler *et al.*, 1989) providing further evidence that the pars distalis is not a significant source of circulating αMSH. Most βEND is likely to be also derived from the pars intermedia although since dexamethasone is able to partially suppress βEND there may be some secretion of this peptide by the pars distalis (Ssewannyana *et al.*, 1989).

Further evidence supporting the concept that the pars intermedia is the major source of αMSH and βEND cosecretion is provided by the suppression of secretion of both peptides, but not ACTH, by the dopamine D2 receptor agonist bromocriptine (Engler *et al.*, 1989). Moreover, circulating αMSH and βEND are dramatically increased after hypothalamo-pituitary disconnection without a corresponding increase in circulating ACTH (Clarke *et al.*, 1986). This suggests that the secretion of these peptides is under inhibitory hypothalamic regulation in the intact animal and is consistent with the model of tonic inhibitory dopaminergic control of the pars intermedia established in other species (Oyarce *et al.*, 1996).

1.2.4.2 Ontogeny of POMC expression in the ovine pituitary gland

POMC mRNA expression is first seen in the fetal sheep pars distalis at day 60 (Yang *et al.*, 1991) and increases to parturition (Challis and Brooks, 1989). Some workers report a decrease in POMC mRNA expression in the last three weeks of gestation and this may reflect increased glucocorticoid feedback from the developing adrenal cortex (Merei *et al.*, 1993). The corticotroph may be unresponsive to glucocorticoids before this stage of development since where the hypothalamus is surgically disconnected from the pituitary in the 100 day fetal sheep, POMC mRNA is unaltered by exogenous cortisol. ACTH storage is increased in the pituitary gland of the sheep nearer parturition and this increased storage may be due to increased negative feedback by cortisol (Challis and Brooks, 1989). However, circulating levels of ACTH increase by more than two fold from 110 days to 147 days of gestation in the fetal sheep (Saphier *et al.*, 1993) in spite of a 20 fold increase in circulating cortisol (Challis and Brooks, 1989) in a similar time frame. This dramatic increase in circulating cortisol suggests increased sensitivity of the adrenal cortex to ACTH and this is confirmed by studies on the fetal sheep demonstrating that the adrenal cortex does indeed become more responsive to ACTH and αMSH and increases in weight exponentially at this stage of development (Hagan and Brooks, 1996). Furthermore the ratio of mature ACTH $_{(1\text{-}39)}$ to larger immunoreactive forms, which are thought to be antagonistic to ACTH at the adrenal cortex, increases to parturition (Saoud and Wood, 1996). The expression of circulating ACTH correlates well with the expression of POMC mRNA. In the sheep, parturition is believed to be dependant on increased fetal circulating ACTH and cortisol immediately prepartum (Challis and Brooks, 1989). Increased ACTH near to term stimulates increased circulating cortisol which in the sheep acts at the placenta to increase the production of estrogens and uterine smooth muscle activity (Liggins, 1974; Saoud and Wood, 1996). The corticotroph secretes ACTH in response to CRF by day 110 of gestation (Challis and Brooks, 1989). Postnatally, pars distalis POMC mRNA expression decreases from the neonate to the adult.

POMC expression in the pars intermedia is detected at a later stage than in the pars distalis. In the pars intermedia ultrastructural evidence for biosynthetic activity is reported by day 70 of gestation (Perry *et al.*, 1982). Soon afterwards, D2 dopamine receptors are expressed and the ovine melanotroph responds to the dopamine agonist, bromocriptine with a two fold decrease in POMC mRNA expression and decreased circulating αMSH by 130 days of gestation (Hagan and Brooks, 1996; Matthews *et al.*, 1996). A corresponding response in the secretion

of ACTH to dopamine agonists and antagonists suggests that the fetal pars intermedia secretes a significant amount of ACTH (Hagan and Brooks, 1996). The pars intermedia may, therefore, have a significant role in the induction of parturition in the sheep.

In the rat, the development of corticotroph and melanotroph function follows a similar temporal order. Expression of POMC mRNA is first detected in the ventral aspect of Rathke's pouch, which becomes the pars distalis at embryonic day 13, two days before it is expressed in the presumptive pars intermedia (Lugo *et al.*, 1989). The rat corticotroph expresses receptors for and is sensitive to stimulation by CRF at embryonic day 15 (Scott and Pintar, 1993). This just precedes the invasion of the median eminence by CRF neuron terminals at embryonic day 16 (Lugo *et al.*, 1989). Soon afterwards the corticotroph begins to process the POMC precursor. The processing enzyme PC2 is abundantly expressed in the corticotroph of the fetal and neonatal rat (Mains and Eipper, 1990) and mouse (Marcinkiewicz *et al.*, 1993) suggesting that processing of POMC by the neonatal corticotroph is more extensive where it occurs, producing POMC products more chacteristic of the melanotroph, during fetal and neonatal life. PC2 expression in the pars distalis is negligable in the adult and the loss of PC2 activity may be associated with suppression by glucocorticoids (Mains and Eipper, 1990). In spite of the more extensive nature of POMC-processing in the fetal compared to the adult corticotroph, efficient processing of POMC does not seem to occur in the rat until late in development. In the neonate only 50% of pars distalis POMC is processed compared to over 90% in the adult (Mains and Eipper, 1990).

In the rat, immunoreactive αMSH is detected in the pars distalis before POMC mRNA is expressed in the pars intermedia (Lugo *et al.*, 1989). In the pars intermedia, the expression of POMC mRNA is first detected at about embryonic day 15, increases steadily to birth and subsequently increases a further four fold to weaning (Hindelang *et al.*, 1990; Lugo *et al.*, 1989). The processing enzymes PC1 and PC2 are first detected in the pars intermedia of the mouse one day after POMC mRNA is first expressed (Marcinkiewicz *et al.*, 1993). In the rat, αMSH is not detected in the pars intermedia until day 19, some time after POMC mRNA expression is first detected (Allen *et al.*, 1984). The neonatal pars intermedia seems to be sensitive to glucocorticoids in the rat, since dexamethasone inhibits the expression of POMC mRNA one day after birth but has no effect ten days later (Scott and Pintar, 1993).

1.2.4.3 Direct innervation and regulation of the pars intermedia

The pars intermedia is much more closely associated with the pars nervosa than with the pars distalis from which it is separated by the residual lumen of Rathke's pouch (Wingstrand, 1966). The melanotrophs are therefore in direct contact with neurons of the hypothalamus which extend nerve terminals into the pars nervosa and regulate the activity of the pars intermedia (Chronwall *et al.*, 1998). Direct innervation of the pars intermedia was first observed in the rabbit (Atwell, 1918) and occurs in many species including the sheep (Chronwall *et al.*, 1998; Danger *et al.*, 1989; Perry *et al.*, 1981). In the rat direct contacts to melanotrophs by dopaminergic and GABAergic neurons have been observed and the degree of individual melanotroph innervation has been implicated in apparent heterogeneity in melanotroph activity (Chronwall *et al.*, 1998).

The nerve terminals of the mammalian pars nervosa exert a regulatory control over the pars intermedia which is principally inhibitory. In the sheep, hypothalamo-pituitary disconnection, which also results in atrophy of the pars nervosa results in increased circulating αMSH (Clarke *et al.*, 1986). Similarly, posterior pituitary denervation in the rat, which abolishes tyrosine hydroxylase activity in the posterior lobe and results in polyurea due to the loss of vasopressin, increases circulating αMSH by two fold (Vecsernyes *et al.*, 1997).

1.2.4.4 Dopaminergic innervation

In the sheep dopaminergic neurons, originating at least partly in the hypothalamic A15 nucleus, infiltrate the pars intermedia and make direct synaptic contacts with individual melanotrophs. Tyrosine hydroxylase immunoreactive neurons are also observed in the pars intermedia and pars nervosa of the porcine pituitary gland (Leshin *et al.*, 1996). Similarly in the rat the pars intermedia is innervated by dopaminergic neurons originating at least partially in the periventricular region of the hypothalamus (Goudreau *et al.*, 1992), specifically the A14 nucleus (Goudreau *et al.*, 1995). The pars intermedia is under tonic inhibitory control by dopamine secreted by these neurons, which acts in the rat by decreasing the responsiveness to β-adrenergic input through specific G-protein negatively-coupled D2 dopamine receptors (Cote *et al.*, 1981; Cote *et al.*, 1982b). The stimulation of these receptors blocks the activity of adenylate cyclase and so reduces intracellular cAMP levels. Dopamine seems to exert the principle role in the regulation of pars intermedia activity in most species studied (Oyarce *et al.*, 1996; Ssewannyana and Lincoln, 1990). The only mammalian exception to this pattern of dopaminergic innervation and inhibitory control documented to date is the rabbit in which glucocorticoids appear to exert the major inhibitory control over the pars intermedia (Schimchowitsch *et al.*, 1994).

In the rat, chronic treatment with the dopamine agonist, bromocriptine, decreases mRNA levels for POMC two to four-fold (Lundblad and Roberts, 1988) and mRNA for many of the enzymes involved in the processing of POMC including PC2, CPE and PAM by approximately two-fold (Bloomquist *et al.*, 1991; Oyarce *et al.*, 1996). Moreover, chronic treatment with haloperidol, a dopamine antagonist, results in an increase in mRNA expression for these enzymes (Bloomquist *et al.*, 1991; Chronwall *et al.*, 1987; Oyarce *et al.*, 1996) while POMC mRNA is increased two to seven-fold (Chen *et al.*, 1983; Hollt *et al.*, 1982a; Lundblad and Roberts, 1988). Circulating βEND is increased by up to five fold after such treatment (Hollt and Bergmann, 1982b). POMC and PC2 mRNA levels generally show a greater sensitivity to dopamine than PC1, CPE and PAM (Oyarce *et al.*, 1996). Moreover N-acetyl-transferase activity may also be regulated by dopamine since it is found that cultured rat melanotrophs secrete a greater ratio of acetylated βEND to non acetylated forms when treated with haloperidol (Ham *et al.*, 1984). Since the processing enzymes are expressed at a level much lower than that of POMC, the regulation of their expression by dopamine may have a large impact on the synthesis of mature POMC-derived peptides (Oyarce *et al.*, 1996). Biosynthetic rates of POMC-derived peptides were found to change even more markedly than mRNA levels in response to such treatments suggesting that dopamine may also exert an inhibitory translational effect (Oyarce *et al.*, 1996). Dopamine antagonists seem to exert a short term biphasic effect where peptide secretion alone is initially increased and then later gene expression is also increased so that stored peptide is replenished. In the longer term regression in the murine pars intermedia is noted after long term treatment with bromocriptine (Beaulieu *et al.*, 1984). Chronic treatment with bromocriptine and haloperidol (over 12 days) were found to have an influence on DNA synthesis (decreased and increased respectively, measured by [^{3}H]thymidine incorporation) suggesting that dopamine suppresses cell proliferation in the rat pars intermedia (Chronwall *et al.*, 1987). In this study a change in the gross morphology of the pars intermedia was noted as a result of changes in the number of melanotrophs due to proliferation. The response to these treatments in POMC expression was more rapid (significant within a few hours) than the proliferative response. Proliferation of melanotrophs has been observed by other workers in the adult rat (Saland, 1981) and immature mice (Llanos *et al.*, 1987).

1.2.4.5 β-Adrenergic innervation

Melanotrophs secrete POMC-derived peptides in response to β-adrenergic input through specific G-protein coupled receptors (Cote *et al.*, 1982a) which are

expressed in the pars intermedia of the rat (Chronwall *et al.*, 1998) and are mediated by increased intracellular calcium (Nemethy *et al.*, 1998). This response is biphasic and is proceeded by a small decrease in calcium (Nemethy *et al.*, 1998). Noradrenaline, the endogenous ligand for β-adrenergic receptors, is synthesised by the enzyme dopamine β-hydroxylase and this enzyme is present in the pars nervosa (Chronwall *et al.*, 1998). Alternatively peripherally circulating catecholamines such as epinephrine from the adrenal medulla may stimulate the secretion of αMSH from the pars intermedia. β-adrenergic receptors also seem to mediate increased αMSH secretion in response to histamine since the β-adrenergic receptor antagonist propanolol abolishes this response (Kjaer *et al.*, 1995; Knigge *et al.*, 1991).

1.2.4.6 GABAergic innervation

The murine pars intermedia, as well as the pars nervosa and median eminence, is also directly innervated by GABA neurons which originate in the hypothalamus (Vincent *et al.*, 1982). The influence of GABA on the melanotroph of the rat is generally inhibitory although there is evidence to suggest that GABA can in the short term actually stimulate αMSH secretion from the melanotroph in vitro (Loeffler *et al.*, 1986; Tomiko *et al.*, 1983) before exerting an inhibitory action. The GABA agonist, muscimol, stimulates the secretion of αMSH in vitro in the short term while GABA inhibits the increased secretion of αMSH resulting from increased potassium ions (Tomiko *et al.*, 1983). The rat pars intermedia expresses the $GABA_A$ receptor which is activated by not only GABA, but also endozepines (natural analogues of benzodiazepines), steroids and barbiturates, although these other ligands bind at different parts of the receptor structure. $GABA_A$ receptor activation by administration of benzodiazepines causes a 25% reduction in pars intermedia POMC mRNA (Garcia de Yebenes *et al.*, 1997).

1.2.4.7 Serotoninergic innervation

In the rat the pars intermedia is innervated by neurons with serotonin immunoreactivity (Autelitano *et al.*, 1989), expresses serotonin receptors and responds with increased αMSH secretion to serotonin (Chronwall *et al.*, 1998; Randle *et al.*, 1983). Where serotonin is experimentally depleted with a neurotoxin, POMC mRNA is increased in the murine pars intermedia and the increase in POMC mRNA associated with stress is abolished (Garcia-Garcia *et al.*, 1997). The possibility exists that serotonin may be principally exerting an effect on the corticotroph since the secretion of βEND is more sensitive to stimulation than that of αMSH after treatment with serotonin agonists or the serotonin reuptake inhibitor, fluoxetine (Carr *et al.*, 1991). Serotonin may be more relevant to the

CRF neuroendocrine system than the pars intermedia, since serotonin stimulates the secretion of CRF from cultured rat hypothalamic neurons in vitro (Hillhouse and Milton, 1989). Furthermore, the immunoreactivity in the pars nervosa of the rat is abolished by fluoxetine, suggesting that serotonin is not synthesised but is internalised by neurons of the pars intermedia (Saland *et al.*, 1987), possibly through a dopamine reuptake mechanism (Chronwall *et al.*, 1998). Moreover the effects of the serotonin agonist, MK-212 on αMSH, but not βEND secretion can be blocked by pretreatment with the dopamine agonist apomorphine (Carr *et al.*, 1991) suggesting that the pars intermedia response to serotonin is likely to be mediated by dopamine. Taken together these reports suggest that while melanotrophs may be capable of responding to serotonin, it is unlikely that serotonin plays a significant part in the direct regulation of pars intermedia activity.

1.2.4.8 Neuropeptide Y (NPY) innervation

There is evidence that NPY neurons innervate the pars intermedia in some species. This is well recognised in some lower vertebrates (Danger *et al.*, 1989) and NPY binding sites are found in follicular-stellate cells of the amphibian pars intermedia (De Rijk *et al.*, 1991). While some reports suggest NPY neurons also occur in the rat pars nervosa and occasionally the pars intermedia (Vanhatalo and Soinila, 1996), other workers have failed to confirm such innervation (Chronwall *et al.*, 1998). It is likely that NPY is less relevant to the mammalian pars intermedia than in the lower vertebrates and that NPY neurons in the pars nervosa, if present, may have a role unrelated to pars intermedia function.

1.2.4.9 Regulation of the pars intermedia by CRF and AVP

The pars intermedia of the sheep, rat and frog responds to CRF with increased secretion of POMC-derived peptides (Fora *et al.*, 1996; Kraicer *et al.*, 1985; Verburg-van Kemenade *et al.*, 1987), and CRF receptors have been localised to murine melanotrophs. However, the murine pars nervosa contains no CRF neurons (Chronwall *et al.*, 1998). It is conceivable that small quantities of CRF may reach the pars intermedia through its vasculature, though this seems unlikely to represent a significant regulatory system since in most species the pars intermedia is poorly vascularised and is not supplied with blood from the portal vessels. The ovine pars intermedia is, however, more vascularised than that of the rat and it is reported that the fetal pars intermedia secretes increased ACTH in response to AVP and CRF (Fora *et al.*, 1996). The expression of the CRF-like peptide, urocortin (Vaughan *et al.*, 1995) has been reported in the pituitary gland of

the rat (Wong *et al.*, 1996), raising the possibility of paracrine stimulation of the secretion of POMC-products through a CRF receptor mediated mechanism.

1.2.5 Peripheral and auto-regulation of pituitary gland POMC expression

1.2.5.1 Glucocorticoid negative feedback

Glucocorticoids reduce the expression and secretion of POMC-derived peptides from the rat corticotroph (Gagner and Drouin, 1985) and the glucocorticoid receptor-ligand complex may act as a trans-acting factor in the inhibition of gene transcription (Autelitano *et al.*, 1989). Additionally glucocorticoids also act in the hypothalamic paraventricular nucleus to reduce the expression of CRF in the ovine fetus (Myers *et al.*, 1992) implicating a negative feedback effect at both hypothalamic and pituitary levels. Glucocorticoids reduce the expression of POMC and secretion of POMC-derived peptides both in the pars distalis and in cultured ovine corticotrophs (Levin *et al.*, 1993). The glucocorticoid receptor is not normally expressed in the mammalian pars intermedia in vivo (Antakly and Eisen, 1984) except in the rabbit (Schimchowitsch *et al.*, 1994). This is confirmed by the observation in the rat that while adrenalectomy increases POMC mRNA expression in the pars distalis, it has no such effect in the pars intermedia (Schacter *et al.*, 1982). However, where rat melanotrophs are isolated from the hypothalamus by autotransplantation to the kidney capsule, they respond to stress with a decrease in POMC gene expression (Iturriza, 1989). This seems to suggest that glucocorticoids can act directly on melanotrophs in isolation from the hypothalamus and this is substantiated by the finding that where the pars intermedia of the rat is isolated from hypothalamic input, glucocorticoid receptor expression develops (Antakly *et al.*, 1985; Seger *et al.*, 1988). This can be reversed by treatment with bromocriptine and dopamine itself suggesting that hypothalamic dopamine suppresses the expression of glucocorticoid receptor in the pars intermedia of this species (Antakly *et al.*, 1987). Moreover the rabbit pars intermedia, the one known mammalian example in which the glucocorticoid receptor is normally expressed, lacks pars intermedia responsiveness to dopamine and possibly even lacks dopamine innervation (Schimchowitsch *et al.*, 1994). There are, however, reports of the presence of immunoreactivity to glucocorticoid receptor in the rat pars intermedia, which is mostly cytosolic, suggesting that the receptor is in an inactive form (Bertini *et al.*, 1989). Since glucocorticoid sensitivity occurs in the pars intermedia of the rat after treatment with the inhibitor of protein synthesis, puromycin, it has been suggested that glucocorticoid receptors are present in the

pars intermedia of the rat under normal circumstances in a form immunocytochemically masked by an endogenous protein inhibitor. It is possible that where glucocorticoid receptors are inactive or inhibited, their conformation does not allow exposure of the DNA-binding motifs of the receptor, to which most antibodies have been raised (Cidlowski *et al.*, 1990). The ovine pars intermedia, however, does not respond to dexamethasone with any change in POMC mRNA expression where hypothalamic influence is removed from the pars intermedia in the HPD sheep (Mercer *et al.*, 1989), thus it is unlikely that glucocorticoid receptor is expressed in the ovine pars intermedia even in the absence of hypothalamic influence.

1.2.5.2 Melanotroph intra-cellular response to stimulation

Melanotrophs of the pars intermedia secrete stored POMC-derived products such as αMSH, in a high frequency pulsatile fashion (Engler *et al.*, 1989; Hagan and Brooks, 1996) in response to cAMP-mediated secretogogues such as noradrenaline (Cote *et al.*, 1982b; Miyazaki *et al.*, 1984). The identity of the specific substrate phosophorylated by cAMP-dependant protein kinase activity and which is responsible for the exocytosis of the secretory granule in the melanotroph has not been identified. There is some evidence that these events are mediated by intracellular calcium signalling since removal of calcium prevents secretion and the calcium ionophore A23187 stimulates secretion without affecting cAMP concentrations (Miyazaki *et al.*, 1984).

Basal αMSH secretion is generally maintained at a high default level but is tonically suppressed by dopamine which exerts its effect through D2 dopamine receptors (Ssewannyana and A, 1990) which are G-protein negatively coupled (Cote *et al.*, 1982b). When stimulated they block the action of adenylate cyclase in catalysing the synthesis of cAMP (Lundblad and Roberts, 1988). A decrease in sensitivity to β-adrenergic stimulation after exposure to dopamine agonists has also been reported (Cote *et al.*, 1981) .

The full cellular repertoire of POMC-derived peptides are likely to be present within the same secretory granules and cosecreted in response to secretagogues although there are reports of differential secretion of particular POMC-products (Randle *et al.*, 1983). Since many POMC-derived peptides are synthesised and mature in the secretory vesicle (Milgram and Mains, 1994) such differential regulation seems unlikely.

1.3 POMC and its products

1.3.1 The POMC molecule

ACTH and αMSH were long known to be produced from a single precursor protein and it was subsequently discovered that βLPH and βEND, which colocalised to the same pituitary cell types, were also produced from the same precursor (Mains *et al.*, 1977). This precursor, POMC, is a 265 amino acid prohormone expressed in both the anterior and the posterior lobes of the ovine pituitary gland and is cleaved into a number of smaller biologically active peptides by the prohormone convertase enzymes in a tissue-specific manner (Figure 1.1). In the pars distalis, POMC is expressed only by a proportion of cells, the corticotrophs. The principle products of POMC processing in these cells are ACTH and βLPH. In the pars intermedia POMC is expressed at a very high level, its mRNA being 10% of all mRNA (Oyarce *et al.*, 1996) produced, by melanotrophs which are the principle cell type of this tissue. There are estimated to be between 20000 and 50000 transcripts for POMC present in the average murine melanotroph (Lundblad and Roberts, 1988; Oyarce *et al.*, 1996). Subsequent processing of POMC by the prohormone convertases in the melanotroph is more extensive than in the corticotroph and βEND, αMSH, CLIP and βCT are among the products of POMC processing in the pars intermedia (Figure 1.3). POMC is expressed in a great many other tissues including the hypothalamus, the amygdala (Garcia-Garcia *et al.*, 1997), pigment cells of the skin and the pancreatic islets of Langerhans where POMC-derived peptides can stimulate insulin secretion through a paracrine action (Borelli *et al.*, 1996). The widespread pattern of POMC expression may reflect that the products of the POMC protein are pleiotrophic in their physiological effects. Extrapituitary POMC is also expressed in immune cells and both leydig and germ cells in the testis where in the rat a mRNA transcript for POMC is produced that is 250bp smaller than the 1100bp transcript expressed in the rat pituitary gland (Autelitano *et al.*, 1989; Smith and Funder, 1988). Similarly in the sheep a 1200bp transcript is detected in the pituitary gland compared to a transcript 100-200bp smaller in the testis, ovary and placenta (Yang *et al.*, 1991). POMC is widely phylogenetically conserved and it is expressed throughout the vertebrates and some invertebrate phyla such as the annelida (Salzet *et al.*, 1997). The murine, porcine, bovine and human POMC genes contain three exons separated by two large introns in the 5' untranslated region and the N-terminal fragment, which are rapidly removed from the primary RNA transcript in the nucleus before transport to the cytoplasm (Castro and Morrison, 1997). Changes in the expression of the primary RNA transcript, because of its transient nature are detectable before changes in mRNA and this phenomenon has been utilised in studies on the temporal nature of POMC gene expression in response to dopamine antagonists. The expression of the primary POMC transcript is increased within

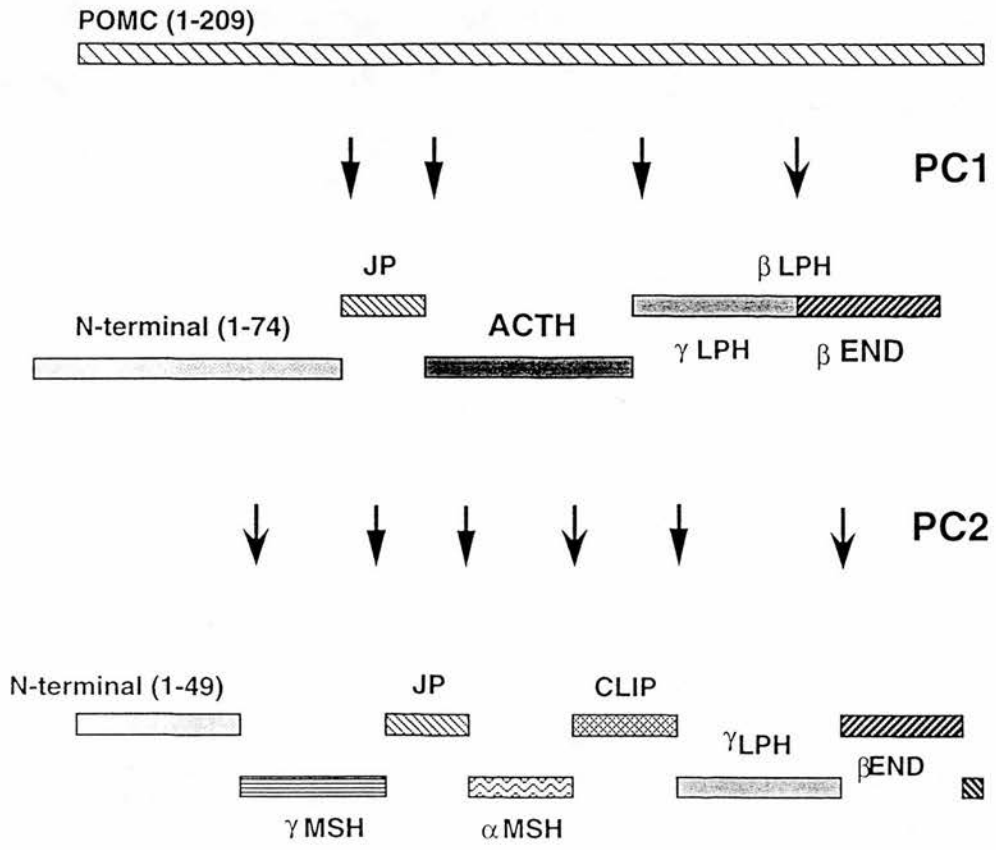


Figure 1.3 The endoproteolytic processing of POMC by PC1 (top) and PC2 (bottom)

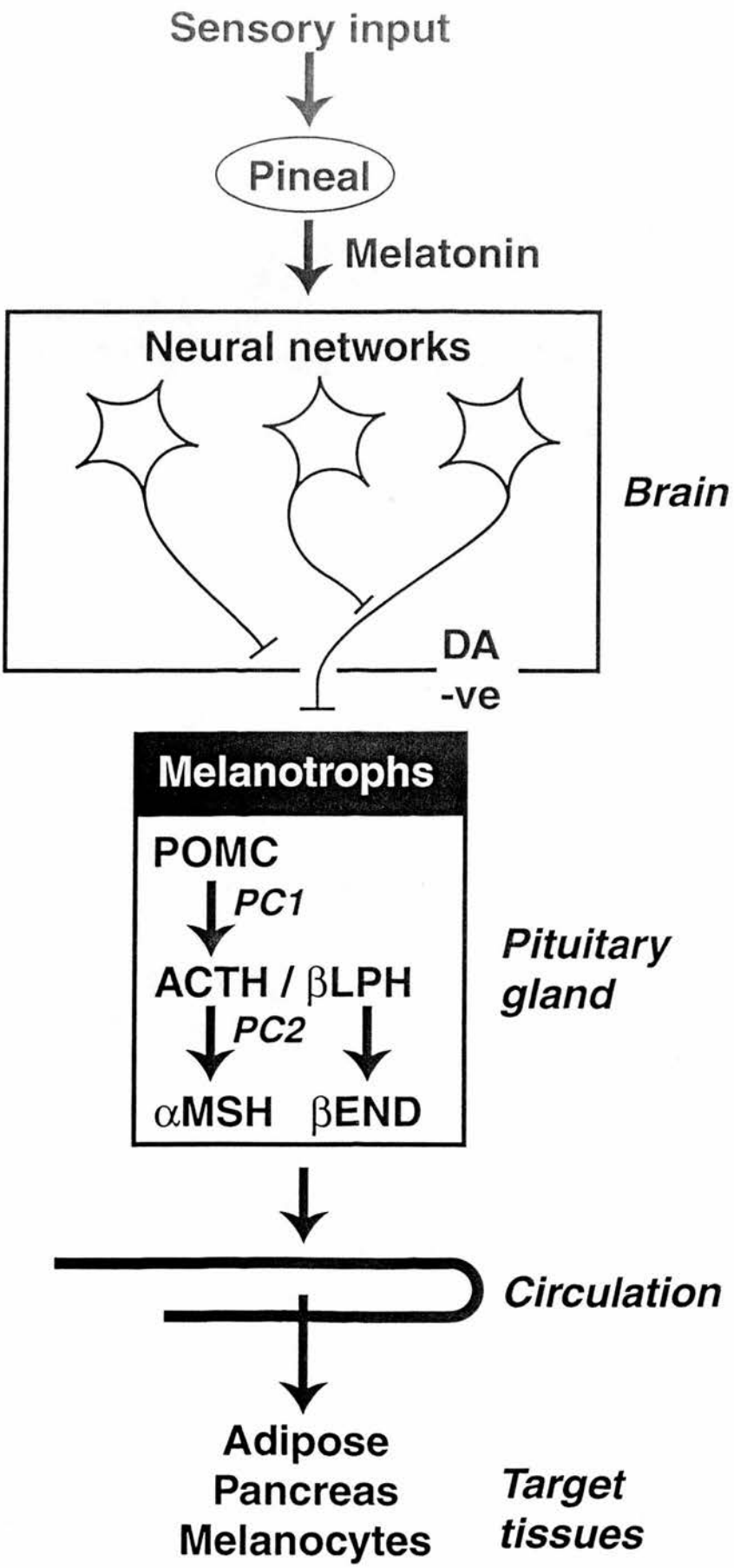


Figure 1.4. Summary diagram illustrating the way in which the hypothalamus may mediate the effects of photoperiod to regulate POMC expression and processing in the melanotrophs of the pars intermedia to dictate the seasonal cycle in αMSH secretion in the sheep.

one hour of haloperidol administration in the rat (Lundblad and Roberts, 1988). Studies on the rat POMC gene in which its 5' untranslated regulatory sequence is fused to a CAT reporter gene reveal that relative to the start of translation site the start of transcription occurs at -95 nucleotides and suppressive regulatory sites occur between -59 and -35. Promoter sequence occurs from -35 to -21 nucleotides (Lundblad and Roberts, 1988). The rat POMC promoter contains at least nine regulatory elements and the synergistic action of all these elements determine the level of POMC gene expression (Castro and Morrison, 1997). Only one POMC gene occurs in all species studied except for a murine pseudogene which is not expressed and probably arose through transposon activity (Lundblad and Roberts, 1988). Moreover, the porcine pars distalis and pars intermedia express identical POMC mRNA transcripts (Gen *et al.*, 1994). Thus, the tissue-specific regulation and processing of POMC is not likely to result from multiple gene copies, rather POMC is processed in a tissue-specific way to yield a number of biologically active products. The nature of these products is determined by the nature and extent of POMC-processing and modification (Eipper and Mains, 1980).

1.3.2 The POMC-derived peptides

1.3.2.1 Adrenocorticotrophic hormone (ACTH)

ACTH is one of the principle secreted products of the corticotroph but is also produced in the pars intermedia as an intermediate in αMSH synthesis. ACTH acts principally on the adrenal cortex where it stimulates the secretion of cortisol. It is thought to be essential in the maturation of the adrenal cortex in the sheep, since hypophysectomy results in adrenal cortical hypotrophy. ACTH induces the prepartum cortisol surge which causes parturition and synchronises the maturation of tissues such as the lungs in preparation for birth (Challis and Brooks, 1989). ACTH may also be an immunomodulator and its secretion is affected by immune status through circulating cytokines (Rivier, 1995) including leukaemia inhibitory factor (Ray *et al.*, 1996). ACTH and glucocorticoids are particularly increased in rats in response to stress such as hypoxia, restraint (Gaillard and Al-Damluji, 1987), pain (Smith and Funder, 1988) and dietary protein deficiency (Jacobson *et al.*, 1997).

1.3.2.2 β-Lipotrophin (βLPH)

There is little literature on the biological actions of βLPH, although it is known to stimulate the production and secretion of steroids such as cortisol and aldosterone from the adrenal gland (Matsuoka *et al.*, 1981; O'Connel *et al.*, 1996).

1.3.2.3 α-Melanocortin (αMSH)

αMSH is a basic 13 amino-acid peptide with a molecular weight of 1663Da. αMSH is derived from ACTH peptide sequence ($_{1-13}$) through endoproteolytic cleavage of the prohormone and subsequent acetylation at the N-terminal serine residue in the N- and 0- positions (Castro and Morrison, 1997). In the ovine pars intermedia, mono-acetylated, diacetylated and des-acetylated αMSH occur in approximately similar amounts (Smith and Funder, 1988). As well as in the pituitary pars intermedia and pars distalis, αMSH occurs in the hypothalamus, thalamus, midbrain, amygdala, the brain stem and peripherally the skin, gut, placenta, testis, ovary and the adrenal medulla (Catania and Lipton, 1993).

αMSH and ACTH exert their biological effects through a number of recently cloned receptors. Five subtypes of the melanocortin receptor have been cloned to date and the tissue distribution and ligand specificity of each is unique (Mountjoy and Wong, 1997). The expression of the type one melanocortin receptor (MC1-R) occurs in adipose tissue, the melanocytes and melanomas of the skin and in macrophages (Mountjoy and Wong, 1997). MC1-R is involved in pigmentation in melanocytes and its primary ligand is αMSH. An endogenous antagonist, the agouti protein, occurs in the mouse which regulates the balance between the production of two melanin pigments, the black eumelanin and yellow phaeomelanin. Inappropriate central expression of agouti coupled with overexpression in the melanocyte results in the abnormalities seen in the yellow obese mouse which has a yellow coat colour and an obese, insulin-resistant phenotype (Lu *et al.*, 1994). The second receptor type (MC2-R) is the classic ACTH receptor, which has a low affinity for αMSH and is involved in the HPA axis. This receptor is expressed in adipose tissue as well as the adrenal gland (Mountjoy and Wong, 1997). The type three receptor (MC3-R) is expressed centrally as well as in placenta, duodenum, the pancreas and the stomach (Mountjoy and Wong, 1997). MC3-R acts as a receptor for γMSH as well as αMSH (Catania and Lipton, 1993).

The type four receptor (MC4-R) is expressed centrally in the brain, spinal cord, sympathetic nervous system and muscle (Mountjoy and Wong, 1997). This receptor subtype is implicated in the central control of appetite since its inactivation in the mouse results in hyperphagia and obesity (Huszar *et al.*, 1997). αMSH and desacetyl-MSH have potent effects in promoting weight gain in white and brown adipose tissue, and muscle in the mouse (Shimizu *et al.*, 1989). However, intra-cerebroventricular administration of αMSH is also reported to reduce food intake in rats while attenuating CRF-induced anorexia (Oohara *et al.*, 1993). This

inconsistency in the response in voluntary food intake to αMSH is complicated by the fact that acetylated and des-acetylated forms appear to have antagonistic effects, acetylated forms having an inhibitory and des-acetylated forms having a stimulatory effect on food intake (Mountjoy and Wong, 1997). In the rat, pars intermedia αMSH is generally acetylated to a greater degree than hypothalamic αMSH (Castro and Morrison, 1997).

The fifth receptor (MC5-R) is expressed in the brain and spinal cord, in skin, exocrine glands, prostate gland, adrenal cortex, spleen, thymus, lymphoid tissues, bone marrow, pituitary gland, testis, ovary, skeletal muscle and adipose tissue (Mountjoy and Wong, 1997; van der Kraan *et al.*, 1998). MC5-R has a more widespread pattern of peripheral expression than any of the other melanocortin receptor subtypes and is particularly highly expressed in the lacrimal gland, preputial gland and harderian gland (van der Kraan *et al.*, 1998).

αMSH stimulates the excretion of sodium ions from the kidney through the increased secretion of atrial natriuretic peptide in the rat (Castro and Morrison, 1997). The genetically spontaneously-hypertensive rat has a phenotype which includes increased pars intermedia POMC gene expression, alterations in central dopaminergic activity and reduced pituitary dopamine content (Autelitano and van den Buuse, 1997). This is consistent with pars intermedia hyperactivity associated with reduced dopaminergic tonic inhibition. Furthermore, chronic salt-loading decreases POMC mRNA levels and causes regression of the pars intermedia of rats (Sharma *et al.*, 1997). These data suggest a role for αMSH in sodium balance and hypertension in the rat. Other MSH peptides such as βMSH and γMSH which occur as a result of specific cleavages of different regions of the POMC molecule (the $\beta LPH_{(41\text{-}58)}$ and N-terminal regions respectively) also have natriuretic properties (Valentin *et al.*, 1993), as described for αMSH.

αMSH has potent antipyretic properties when administered centrally or peripherally and endogenous αMSH may play a role in the regulation of fever. αMSH content in certain brain regions increases in fever and circulating αMSH also increases in response to pyrexia (Catania and Lipton, 1993). αMSH is capable of suppressing local inflammation and hypersensitivity reactions (Catania and Lipton, 1993; Tatro, 1996). This immunoregulatory role of αMSH is probably its most phylogenetically ancient function. POMC is expressed by invertebrate immunocytes (Salzet *et al.*, 1997) and has immunoregulatory properties in invertebrates (Tatro, 1996).

In the human, circulating αMSH decreases after birth probably because of the dramatic involution of the pars intermedia after which only a few isolated αMSH immunopositive cells remain in the adult (Mauri *et al.*, 1993).

1.3.2.4 β-Endorphin (βEND)

βEND is one of the three endogenous opioid receptor ligands, which also include enkephalin and dynorphin. The opioid receptors, specifically μ, δ and κ (Morley and Levine, 1983) were identified before their ligands as the binding sites for exogenous opioids such as morphine. Like morphine, endogenous opioids have powerful analgesic properties.

For some years there has been interest in βEND as a potential factor governing food intake and metabolism. There is good evidence in both rodents and humans that altered central and peripheral levels of βEND are associated with obesity, eating disorders and stress (Mercer and Holder, 1997). Genetically obese mice (ob/ob) and rats (fa/fa) are found to have a higher pituitary content of βEND than lean strains (Margules *et al.*, 1978). Furthermore, periods of increased food intake in rats correlate with increased circulating immunoreactive βEND (Davis *et al.*, 1983). There is some evidence that βEND acts as an insulin secretagogue in the pancreas (Ipp *et al.*, 1978; Reid and Yen, 1981), although a role for βEND on insulin secretion is not consistently observed (Fatouros *et al.*, 1995). Circulating βEND is correlated negatively with circulating glucose concentrations, but not with circulating insulin, suggesting that βEND is involved with glucose metabolism and regulation in an insulin independent way (Fatouros *et al.*, 1995).

In humans, elevated basal levels of circulating βEND are associated with obesity (Balon-Perin *et al.*, 1991; Genazzani *et al.*, 1986; Giugliano and Lefebvre, 1991) and pancreatic β-cell response to βEND is increased in such subjects (Cozzolino *et al.*, 1995; Giugliano and Lefebvre, 1991). Since the adult human pituitary does not contain a distinct neuro-intermediate lobe, and this elevated βEND level is not abolished by dexamethasone, it is unclear whether this plasma βEND is derived from the pituitary.

In the sheep, where hypoglycaemia is induced by an insulin bolus, ACTH, βEND and αMSH are secreted into the peripheral blood. The delay in this response suggests that insulin is not having a direct effect on the secretion of POMC-derived peptides from the pituitary gland (Engler *et al.*, 1988) and that hypoglycaemic stress itself may be the major stimulator to POMC-derived peptide secretion.

Most of the βEND produced by the ovine pars intermedia is of an acetylated form (mostly acetylated $\beta END_{(1\text{-}27)}$) (Smith and Funder, 1988), compared to only 25% acetylation in the ovine pars distalis (mostly $\beta END_{(1\text{-}31)}$) (Castro and Morrison, 1997). Acetylated βEND is thought to be inactive as an opioid receptor ligand (Smith and Funder, 1988). Furthermore it is unlikely that peripheral βEND can cross the blood brain barrier (Mercer and Holder, 1997). It may be that altered levels of peripheral βEND reflect altered central levels and are altered in concert

with bioactive αMSH. It is possible that the secretion of βEND by the pars intermedia is of secondary importance to the cosecretion of αMSH.

1.3.2.5 Corticotrophin-like intermediate lobe peptide (CLIP)

CLIP is a peptide derived from the C-terminal portion of ACTH by endoproteolytic processing in the pars intermedia (Figure 1.3) and its name reflects the fact that the shared peptide sequence often results in cross-reaction with antisera directed against ACTH. CLIP can be extensively modified by post-translational exopeptidase activity, glycosylation and phosphorylation. In the rat many forms of CLIP occur due to the occurrence of different combinations of these modifications (Smith and Funder, 1988). The biological significance of the many forms of CLIP is not well understood.

1.3.2.6 β-Cell tropin (βCT)

βCT, like CLIP, is derived from the processing of ACTH in the pars intermedia. This peptide was first isolated from the plasma of genetically obese (ob/ob) mice and is secreted by the pars intermedia. In studies on perfused pancreas, βCT appears to be an insulin secretagogue in the mouse and rat (Beloff-Chain *et al.*, 1983; Billingham *et al.*, 1982) and has been shown to have insulin-like lipogenic properties in adipose tissue (Watkinson and Beloff-Chain, 1984). Plasma βCT levels have been found to be higher in certain genetically obese strains of mice and rats when compared to lean animals (Morton *et al.*, 1991). This peptide is also detected in human plasma (Salvatoni *et al.*, 1986).

POMC-derived peptides are expressed within the pancreatic islets and their secretion is stimulated by glucose at high concentrations (Borelli *et al.*, 1996). It is therefore possible that pituitary-derived βCT is not directly involved in the control of pancreatic function, but that its effects on the pancreas may implicate the existence of a paracrine mechanism controlling insulin secretion which is mediated by locally synthesised POMC-derived peptides.

1.3.2.7 Other POMC-derived peptides

In addition to the previously-described peptides, the cleavage of POMC in the pars intermedia yields other peptides whose physiological function is obscure, including the 74 amino-acid residue N-terminal fragment which is cleaved to produce γMSH, C-terminal amidated joining-peptide and a smaller 49 amino-acid N-terminal fragment (Smith and Funder, 1988) (Figure 1.3).

1.4 POMC processing and secretion

During POMC translation, the signal peptide at the N-terminus directs POMC to the lumen of the endoplasmic reticulum where the signal sequence is rapidly removed. The nascent POMC peptide is then transported to the golgi stack where it is glycosylated and phosphorylated at specific sites (Smith and Funder, 1988). It is here and subsequently in the immature secretory granule that the endoproteolytic processing of POMC occurs. The endoproteolytic processing of POMC determines the combination of biologically active products liberated from the prohormone and is catalysed by the prohormone convertases, specifically PC1 and PC2 in the melanotroph (Benjannet *et al.*, 1991; Thomas *et al.*, 1991) (Figure 1.3). A similar pattern of POMC processing to that of the pars intermedia is also observed in the hypothalamic arcuate nucleus (Smith and Funder, 1988). PC1 and PC2 are ubiquitous enzymes involved in the processing of a large number of other prohormones (Castro and Morrison, 1997; Smeekens *et al.*, 1992). These include POMC, prorenin, proinsulin, proenkephalin, prosomatostatin, proglucagon, proparathyroid hormone, progastrin, procalcitonin, proCRF and proneurotensin (Castro and Morrison, 1997). The prohormone convertases are part of a large family of subtilisin-like endoproteases expressed throughout the animal kingdom and in yeasts. This family includes yeast Kex2 (Mizuno and Matsuo, 1984), the constitutive mammalian endoprotease Furin (Bresnahan *et al.*, 1990), PC4 and PC5 which are expressed in the testis and ovary respectively (Seidah and Chretien, 1994), PC7, expressed in the colon and lymphoid tissues (Seidah *et al.*, 1996) and PACE4, expressed by somatotrophs, lactotrophs and corticotrophs in the rat (Johnson *et al.*, 1994).

These proteases, while more substrate-specific (Lindberg, 1991), share functional and sequence homology to bacterial subtilisin-like enzymes (Seidah and Chretien, 1994), from which they are likely to be phylogenetically derived. Kex2 was the first characterised of these enzymes, and is a membrane-anchored calcium-dependent endoprotease which produces the yeast α-mating factor from a precursor and possesses a distinct catalytic domain containing a Asp-Ser-His amino acid sequence common to all the prohormone convertases (Castro and Morrison, 1997). All known mammalian prohormone convertases possess an RGD (Arg-Gly-Asn) motif which is a recognition sequence for proteins of the extracellular matrix, although the significance of this motif to the prohormone convertases is unknown (Seidah and Chretien, 1994). The catalytic domain is invariably followed C-terminally by a P-domain without which the enzyme fails to mature and may be important in conformational stabilisation (Lindberg, 1991). At the C-terminus each prohormone convertase has a unique sequence, including in Kex2 the trans-

membrane domain (Seidah and Chretien, 1994). PC1 and PC2 differ from Kex2 in not usually being membrane-bound, although there are reports of some membrane bound forms occurring in the secretory granules of the mouse and the cow (Kirchmair *et al.*, 1992). Different immunoreactive forms of PC1 and PC2 are detected by immunoprecipitation and these are likely to represent endoproteases at different stages of maturation.

All prohormone convertases cleave proteins at specific paired basic amino acid residues, and possibly at single arginine residues, in common with related enzymes (Benjannet *et al.*, 1991; Seidah and Chretien, 1994). Prohormone sequence at sites other than paired basic amino acid residues are also relevant to endoproteolytic cleavages since protein conformation may make these residues more or less stearically exposed to prohormone convertase activity (Jung *et al.*, 1993). PC1 and PC2 are active only late in the secretory pathway and are dependent on acid pH for maximal activity (Jean *et al.*, 1993; Shennan *et al.*, 1991). PC1 activity is likely to precede PC2 activity in the secretory pathway, since it is less sensitive to low temperature which inhibits vesicle transport of secretory products to later stages of the pathway (Milgram and Mains, 1994; Paquet *et al.*, 1996). Endoproteolytic processing is followed by exoproteolytic removal of amino acid residues, α-amidation and acetylation. These modifications, while apparently more subtle than those of the prohormone convertases, have profound effects on the biological potency of the mature peptide. α-Amidation of αMSH, for example, which reduces the C-terminal glycine residue to an amide group, is necessary for biological activity and prevents intracellular degradation (Smith and Funder, 1988).

PC1 and PC2 are restricted to neuroendocrine and endocrine tissues and are expressed in bovine (Kirchmair *et al.*, 1992), murine (Benjannet *et al.*, 1991) and amphibian pituitaries (Kurabuchi and Tanaka, 1997). In the rat and mouse, PC1 occurs in the pituitary pars distalis and the pars intermedia whereas PC2 is mainly confined to the pars intermedia (Benjannet *et al.*, 1991). Similarly in the frog, PC1 and PC2 have been localised at the ultrastructural level in the pituitary gland where PC1 occurs mainly in the corticotrophs of the pars distalis while PC2 is mostly expressed in the amphibian pars intermedia and pars nervosa. PC1 immunoreactivity colocalises with secretory granules containing ACTH immunoreactivity, whereas PC2 colocalises down to the ultrastructural level with αMSH in the pars intermedia (Kurabuchi and Tanaka, 1997). The dependence on PC2 for αMSH biosynthesis is further strengthened by the observation that PC2 is localised to human anterior pituitary tumours producing CLIP (Vieau *et al.*, 1994), which is cosynthesised with αMSH.

1.4.1 The secretion of POMC-derived peptides

The events that occur in the processing and secretion of POMC rely on a complex molecular machinery acting in concert. Two modes of exocytotic secretion are observed. In the first secretory products are constitutively synthesised and secreted and hence this is known as the constitutive secretory pathway. The products of POMC-processing are secreted via the second mode of secretion, the regulated secretory pathway, which involves the concentration of secretory products into specialised vesicles or secretory granules. These granules store the secretory products, often for long periods of time, until their release following intracellular second messenger mediated hormonal stimulation (Burgess and Kelly, 1987). The processing of POMC is Ca^{2+} and acidic pH dependant, limiting POMC processing to the trans-golgi network and secretory granules (Jean *et al.*, 1993; Shennan *et al.*, 1991). The secretory pathway occurs over a pH gradient, a total decrease of about 1pH unit across the golgi stack (Halban and Irminger, 1994), which is probably important for transport and sorting of secretory products. Associated with maturation of secretory granules, there is a concentration of secretory products, often into an electron-dense inert aggregate and further acidification of the granular contents occurs (Halban and Irminger, 1994). The concentration of secretory products in the secretory granule is estimated to be 20-200 fold that of the golgi (Burgess and Kelly, 1987). These granules are subject to long term storage, and experiments on β-cell insulin secretion suggest that more recently formed granules are preferentially secreted before older ones (Rhodes *et al.*, 1987), although the functional significance of this mechanism is unclear. In the sheep βEND and αMSH seem to be cosecreted suggesting a common secretory granule (Lincoln and Baker, 1995).

1.4.2 PC1

PC1 is involved in the processing of several proproteins including POMC. The mouse PC1 gene contains 14 introns and shares considerable homology to PC2 in the protein level, particularly in the N-terminal catalytic region (Lindberg, 1991). From N-terminal to C-terminal, the proPC1 peptide sequence consists of a pro-region removed during PC1 maturation, a catalytic domain containing the characteristic Asp-Ser-His amino acid triplet, a P-domain containing the characteristic RGD sequence, the unique C-terminal sequence and finally a region of amphipathic helices which may be involved in membrane anchorage (Jung *et al.*, 1993). PC1 commonly cleaves peptides at Arg-Arg, Lys-Arg (to produce ACTH and βLPH and weakly to cleave βLPH to produce βEND, (Benjannet *et al.*, 1991; Jung *et al.*, 1993; Smith and Funder, 1988). PC1 is calcium-dependent and is

optimally active at pH 5.5-6.0 (Jean *et al.*, 1993; Rufaut *et al.*, 1993; Seidah and Chretien, 1994; Zhou and Lindberg, 1993). The acid pH and calcium requirements are consistent with the observation by that most PC1 mediated endoproteolytic activity occurs in immature secretory granules (Tanaka *et al.*, 1997).

The prohormone convertases are themselves produced as proenzymes or zymogens. The maturation of the prohormone convertases is likely to provide an important mechanism ensuring that these enzymes are activated at an appropriate stage of the secretory pathway. Like POMC, the endoproteases PC1 and PC2 both contain internal dibasic paired amino acids which act as sites for internal cleavage autocatalysis. PC1 catalyses the removal of its own proregion in the endoplasmic reticulum (Goodman and Gorman, 1994; Kirchmair *et al.*, 1992; Rufaut *et al.*, 1993) and is then glycosylated in the golgi stack (Lindberg, 1991), conferring stability to the enzyme (Benjannet *et al.*, 1993). PC1 is further modified in the golgi stack increasing the activity of the enzyme (Jutras *et al.*, 1997; Lindberg, 1991).

1.4.3 PC2

The human PC2 gene contains 11 introns and is similar at the peptide level to PC1 but possesses a unique C-terminal region, after the P-domain (Lindberg, 1991). As in proPC1, proPC2 possesses a region of amphipathic loops at its C-terminal which may be involved in membrane anchorage (Jung *et al.*, 1993). PC2 catalyses the cleavage of ACTH at the Lys-Lys cleavage site to produce αMSH and CLIP (Friedman *et al.*, 1996; Smith and Funder, 1988). PC2 is calcium-dependent and is most active at pH5.5 when transiently expressed in Xenopus oocytes (Lamango *et al.*, 1996; Shennan *et al.*, 1991). The slightly lower pH optimum for PC2 compared to PC1 is consistent with an activity later in the secretory pathway. PC2 matures via an autocatalytic mechanism (Shennan *et al.*, 1995). Autocatalysis of PC2 requires low pH and is effectively blocked by low temperature, suggesting maturation in a later secretory compartment than PC1 (Milgram and Mains, 1994). The slower and later maturation of PC2 (Seidah and Chretien, 1994; Shennan *et al.*, 1995; Zhou and Mains, 1994) may ensure the sequential processing of POMC in the pars intermedia. The maturation and activity of PC2 is thought to be regulated by the action of its binding protein, 7B2. This protein bears homology to both chaperonins and bacterial subtilisin inhibitors (Benjannet *et al.*, 1995; Braks and Martens, 1994).

1.5 Predictions and aims of this study

1.5.1 Seasonal cycle in αMSH in the sheep

The seasonal cycle in circulating αMSH in the sheep results from the long term activation and inactivation of the pars intermedia as an adaptation to life in a seasonal environment. A generalised model for the way in which the environment acting through sensory cues may regulate the pars intermedia is illustrated in Figure 1.4. This proposed that cues, particularly photoperiod acting through melatonin, dictate the functional activity of neural networks in the hypothalamus which govern the synthesis and secretion of POMC-derived products from the pars intermedia. Since the pars intermedia is under predominant inhibitory tone through dopamine, it is presumed that exposure to long days enhances dopamine activity to suppress the secretion of αMSH as observed in spring and summer, while exposure to short days reduces dopamine activity to promote the secretion of aMSH as observed in autumn. These changes in αMSH act in turn on the multiple target tissues (adipose, pancreas, melanocytes) to induce the seasonal responses.

1.5.2 Model of coordinated control

The cycle in the adult sheep provides a novel model in which to study the dynamic regulation of POMC expression and processing in the pars intermedia. There have been no studies of the cellular events associated with this cycle. Consequently, the aim of the studies presented in this thesis was to investigate the changes in POMC expression and processing in relation to various physiological treatments and manipulations- season, photoperiod, hypothalamic disconnection and treatment with dopamine. Most of the material was collected from Soay sheep which express a particularly marked seasonal cycle in pars intermedia activity similar to the ancestral Mouflon. The prediction was as follows:

Long term changes in the secretion of POMC-derived peptides in the adult sheep will be the result of coordinated regulation of POMC, PC1 and PC2 gene expression.

1.5.3 Layout of the thesis

The thesis begins with a description of general methods used in the studies including the development of specific molecular probes (Chapter 2). The general anatomy of the pituitary gland in the Soay sheep is presented for comparison with other breeds of sheep and with particular emphasis on the cell types expressing POMC, PC1 and PC2 (Chapter 3). The data with respect to season, photoperiod and hypothalamic control, and influence of dopamine are presented in chapters 4 to 6 respectively. The final chapter aims to integrate the results.

Chapter 2

General Materials and Methods.

2.1. Animals and treatments

2.1.1. Animals

Animals used for the experiments were adult Soay sheep bred in Fife, Scotland (56°N), and housed outdoors in Fife or indoors in the Marshall Building in Roslin, Midlothian. Originating from St Kilda in the North Atlantic, the Soay sheep is a feral breed similar to the wild Mouflon sheep from which all domesticated sheep are derived. Like the wild sheep the Soay displays pronounced seasonally-regulated changes in pelage and food intake as well as reproduction (Argo and Smith, 1983; Lincoln, 1991). Seasonal cycles in αMSH and βEND are well characterised in the Soay sheep (Ebling and Lincoln, 1987) and as such this breed is an appropriate experimental model in which to study the seasonal regulation of the POMC-processing and secretory system. At the end of each experimental regime, animals were killed by sodium pentobarbitone intravenous overdose. For the Northern analyses and to validate some of the methods, material was also obtained from adult Scottish Blackface sheep held at the Moredun Research Institute, Midlothian.

2.1.2. Treatments

The specific experimental treatments are described in more detail in the relevant chapters. Generally, where housed indoors, animals were maintained under an alternating artificial lighting regimen of 16 weekly periods of long (16L: 8D) and short (8L: 16D) days. During the light phase, light intensity was maintained at 160 lux at the animals head level. Light intensity during the dark phase was maintained at less than 5 lux by dim red lighting. Such alternating photoperiods are able to induce and entrain long term cycles in reproductive activity, food intake and pelage growth similar to those seen in animals living outdoors (Lincoln and Baker, 1995; Lincoln and Clarke, 1994). Animals maintained indoors were penned individually with visual and tactile contact between neighbours and were fed on a constant diet of dried grass nuts with hay and water *ad libitum*. Where animals lived outdoors they were maintained in grass paddocks under natural photoperiod and received

supplementary feeding of commercial sheep nuts and hay during the winter (December to April). Animals were allowed free access to food and water throughout all experimental regimes.

2.2. Chemicals and suppliers

Molecular biology grade chemicals were obtained from Sigma, (Poole, UK) and IBI, (Cambridge, UK). All radiolabelled nucleotides were obtained from Dupont NEN, (Stevenage, UK). Enzymes were purchased from Boehringer Mannheim, (Buckinghamshire, UK) or Promega, (Southampton, UK). Phenol/chloroform was purchased from Camlab, (Cambridge, UK) and was pre-buffered with Tris, pH 8.0. Autoradiography products were obtained from Eastman Kodak (Rochester, NY, USA), supplied by Sigma.

2.3. RNA extraction and separation

2.3.1. Dissection of tissue

The pituitary gland and basal hypothalamus were removed shortly after the sheep were culled. The rostral part of the pituitary gland was fixed in Bouins reagent for five hours and then transferred to 70% ethanol before paraffin embedding for histology. The caudal part of the pituitary gland was used for RNA extraction. Within this more distal portion, the pars intermedia and pars nervosa were dissected from the pars distalis and both were snap frozen on dry ice and stored at -70°C until RNA extraction as described below. In some animals, the medio-basal hypothalamus posterior to the optic chiasm and anterior to the mammilary bodies, the latero-ventral hippocampus and cerebellum was snap-frozen for RNA extraction as described for the pituitary gland.

2.3.2. Extraction of RNA from fresh tissue

RNA was extracted using a commercially available guanidinium thiocyanate-based extraction reagent (Chomczynski and Sacchi, 1987), Tri-reagent (Sigma), according to manufacturers instructions, with appropriate adjustment of volumes. The tissue in Tri-reagent was homogenised for approximately 30 seconds until completely dissociated allowing approximately 10ml Tri-reagent per gram of tissue. To this homogenate, 0.2 volumes of chloroform was added and the mixture shaken vigorously for 15 seconds. The mixture was allowed to stand at room temperature and then centrifuged at 12000g for 20 minutes at 4°C. The upper aqueous layer

containing the RNA was transferred to a fresh tube and the RNA was precipitated with 1 volume of cold (-20°C) isopropanol. The RNA was then pelleted by centrifugation at 12000g for 20 minutes at 4°C. Following removal of the supernatant, the RNA pellet was washed in 75% ethanol and then dissolved in RNase-free water by warming to 65°C for 10min. RNA isolated in this way was then stored at -70°C.

RNA was scanned at 260 and 280nm on a spectrophotometer. The 260:280 ratio for each sample was calculated to give an estimation of the purity of the RNA. A ratio of 1.6-1.8 was taken to be pure. The concentration of the RNA was calculated from the 260nm value where an optical density of 1.0 is equal to 40μg/ml RNA.

2.4. Tissue fixation and processing

2.4.1. Fixation

Fixation of the tissue was achieved by submersion in Bouins reagent (Appendix I). The tissue was fixed in Bouins' fluid for 5 hours and transferred to 70% ethanol for storage before processing and wax-embedding, as described below, for immunocytochemistry and *in situ* hybridisation.

2.4.2. Processing and sectioning of tissue

Tissue was processed through a graded series of alcohols in an automatic 2LE Processor (Shandon Scientific Limited, Cheshire, UK) using a standard 20 hour cycle and embedded in paraffin wax. Glass microscope slides to be used for *in situ* hybridisation were washed, dried and baked at 300°C for 8 hours to remove latent RNase activity. All slides were subsequently washed in a 0.25% solution of 3-aminopropyl triethoxysilane (TESPA, Sigma) in acetone (BDH Ltd, Poole, UK), followed by a wash in acetone and finally rinsed in filtered distilled water and dried. Paraffin wax embedded tissue was sectioned to a thickness of 5μm for *in situ* hybridisation, and 3μm for immunocytochemistry using a hand operated "820" Spencer Microtome (American Optical Corporation) and a D-profile knife. Sections were floated on water, RNase-free for *in situ* hybridisation, transferred onto the treated slides and dried overnight before use.

2.4.3. Histological staining of sections

Haematoxylin and eosin staining (Bancroft and Stevens, 1982) was used for general histological characterisation of the pituitary gland. Briefly, sections were

cleared in histoclear (National Diagnostics, Manville, NJ, U.S.A.) and rehydrated in a decreasing series of alcohols. Sections were stained with haematoxylin (BDH), differentiated in acid-alcohol and blued in Scott's tap water. Finally, sections were dehydrated, cleared in xylene and coverslips were mounted with pertex (Cellpath, Hemel Hempstead, UK).

Tissue sections subjected to immunocytochemistry and *in situ* hybridisation were counterstained using haematoxylin alone after the procedure, differentiated in acid-alcohol and blued in Scott's tap water. Finally, sections were dehydrated, cleared in xylene and mounted as above.

2.5. Immunohistochemistry

2.5.1. Tissue pretreatment and primary antibody

Tissue sections prepared as described in section 2.4 and mounted onto glass slides were cleared in histoclear for approximately 10 minutes and rehydrated in decreasing concentrations of alcohol (100%, 96% and 70% respectively). The tissue sections were placed in 3% hydrogen peroxide in methanol for 30 minutes to reduce endogenous peroxidase activity where a peroxidase detection method was used. Where the alkaline phosphatase detection system was used this step was omitted. Slides were then washed in Tris-buffered saline (TBS) pH7.4 for 5 minutes and the tissue sections incubated with normal serum from the species in which the secondary antibody was raised (usually swine or rabbit), diluted 1:5 in TBS for 30 minutes at room temperature. After this incubation, the normal serum was removed carefully from the sections with a tissue and replaced with diluted primary antibody (in 1:5 normal swine serum: TBS). The slides were covered with Gelbond film (Flowgen, Rockland, ME, USA), hydrophilic side down and tissue was then incubated with the antibody overnight at 4°C in a humidified chamber. The following day, excess antibody was removed by two 5 minute washes with TBS.

2.5.2. Secondary antibody and Horseradish Peroxidase detection system

Secondary antibodies for immunocytochemistry were obtained from Dako (Glostrup, Denmark). Immunocytochemistry used the horseradish peroxidase detection system using a kit purchased from Dako. The secondary antibody for the horseradish peroxidase detection system was biotinylated for subsequent conjugation to avidin-linked peroxidase enzyme complex. Secondary antibody was diluted 1:500 in TBS and incubated on the tissue sections for 30 minutes. Excess

antibody was again removed by 2x 5 minute washes with TBS. Sections were incubated in the presence of avidin-horseradish peroxidase complex (Dako, Glostrup, Denmark) for 30 minutes. This bound the biotinylated secondary antibody and formed a complex which served to amplify the signal obtained from the primary antibody by acting on a substrate (DAB and hydrogen peroxide, Sigma) which was subsequently applied to the sections and produced a coloured product. The horseradish peroxidase complex solution was prepared according to the suppliers instructions (Dako) in 0.05M Tris/HCl pH7.6 at least 20 minutes before use. Excess AB complex was removed by 2x 5 minute TBS washes before bound antibody was visualised with a solution of 225μM DAB in 0.05M Tris/HCl, pH7.6 containing 0.01% hydrogen peroxide.

After development of the colour reaction sections were washed in water, stained with haematoxylin, dehydrated and mounted with coverslips. Where attempts to quantify immunocytochemical staining intensity were made, sections were examined without prior knowledge of treatment group and staining intensity was graded from zero (antigen not detectable) to five (heavy immunostaining).

2.5.3 Double immunocytochemical staining for ACTH and LHβ

Double immunocytochemistry on single tissue sections was carried out to visualise the spatial relationship between two secretory cell types, specifically corticotrophs and gonadotrophs. Immunocytochemistry for LHβ was performed using the horseradish peroxidase detection system as previously described but was immediately followed by immunocytochemistry for ACTH using an alkaline phosphatase procedure illustrated in Figure 2.1 (right). The secondary antibody for this procedure was non-biotinylated sheep anti rabbit immunoglobulins (SAR; Sigma). SAR was diluted 1:60 in TBS and incubated on the sections for 30 minutes. This was followed by 2x 5 minute washes with TBS to remove excess, unbound antibody. Sections were incubated for 30 minutes in rabbit alkaline phosphatase anti-alkaline phosphatase (APAAP, Dako) diluted 1:60 in TBS followed by 2x 5 minute washes in TBS. Sections were then equilibrated in buffer containing 0.1M Tris pH9.5, 0.1M NaCl and 50mM $MgCl_2$, for 2 minutes. Detection of antigen was then achieved using the fast blue substrate (Sigma) according to the manufacturers instructions.

After development of the colour reaction sections were washed in water and mounted with an aqueous mounting medium since fast blue is soluble in alcohol. Sections treated in this way were not counterstained.

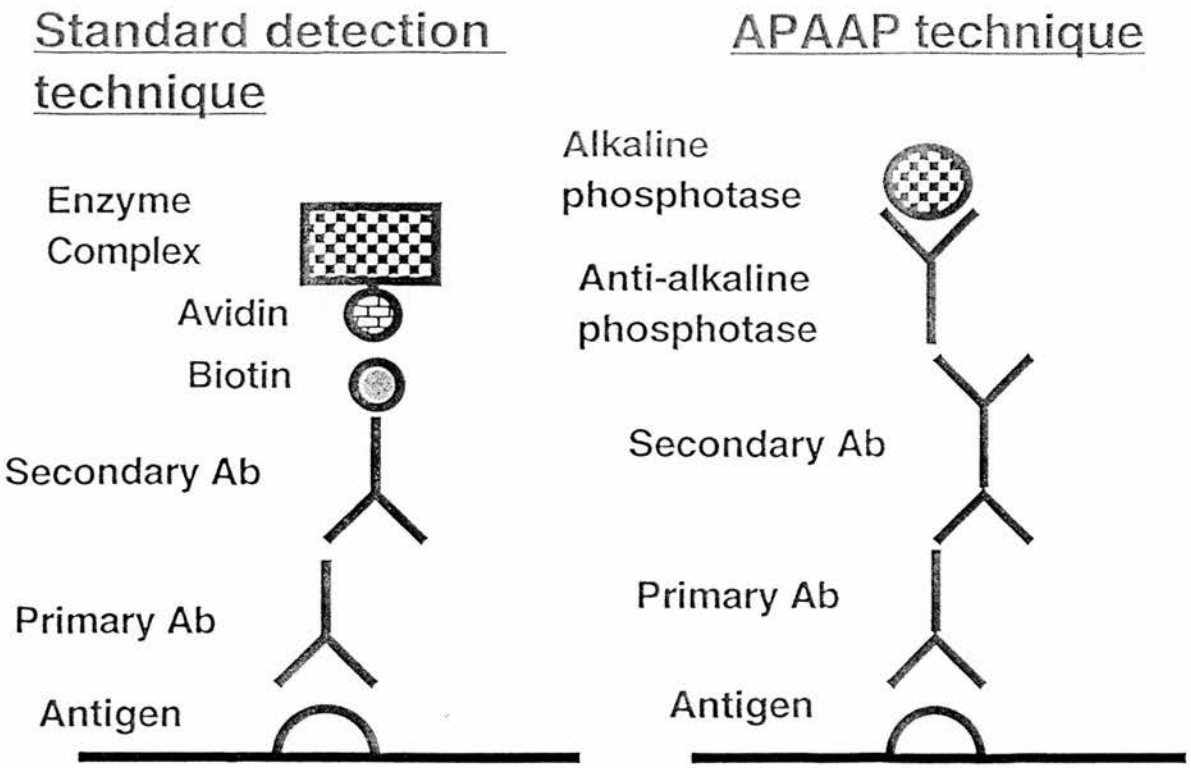


Figure 2.1 Immunocytochemical techniques, standard avidin-biotin HRP or AP procedure (left) and APAAP procedure (right).

2.5.4 Antisera

Specific antibodies were used to identify cell types and tissues within the pituitary gland and access cell proliferation by detection of specific cell proliferation markers. Anti-C-terminal ovine αMSH rabbit polyclonal antibody (provided by Dr Brigette Baker, University of Bath, UK) was used to identify melanotrophs and the pars intermedia at a dilution of 1:1000. The αMSH antibody crossreacts at 70% with ovine des-acetylated αMSH and 0.15% with ACTH. A polyclonal rabbit antibody against ovine CLIP was used for the detection of ACTH (provided by Professor Leslie Rees, St Bartholomews Hospital, London, UK) and was used at a dilution of 1:500. Since this antibody was raised against the N-terminal of CLIP which is also the N-terminal of ACTH, it is 100% cross-reactive with ACTH. Rabbit polyclonal anti-ovine LHβ antibody (provided by NIDDK) was used to identify gonadotrophs at a dilution of 1:3000. Rabbit polyclonal anti-ovine

prolactin antibody (provided by NIDDK) was used to identify lactotrophs at a dilution of 1:5000.

Rabbit polyclonal anti-mouse PC1 antibody (raised against C-terminal segment 629-726) was used at a dilution of 1:600 and rabbit polyclonal anti-mouse PC2 antibody (Raised against mouse PC2 segment 529-637) was used at a dilution of 1:1000 to localise these enzymes to specific cell types. Both the PC1 and PC2 antisera were provided by Dr Mieczyslaw Marcinkiewicz (IRCM, Montreal, Canada). These antisera have been validated in immunocytochemistry on mouse tissue by preabsorbtion with synthetic antigenic peptides (Marcinkiewicz *et al.*, 1993).

The antibody used to identify proliferating cells was monoclonal anti-PCNA (Novacastra, Newcastle upon Tyne, UK) at a dilution of 1:100. PCNA is a 36kDa protein which is highly conserved between species. Since this antibody was raised against mouse PCNA the affinity of the antibody for the sheep protein was tested by preliminary immunocytochemistry in sheep testis. Since spermatogenesis involves repeated meiotic divisions this tissue was used as a positive control. Immunocytochemistry for both these antigens revealed abundant proliferative activity in the sheep testis, as expected.

2.5.5 Control of antibody specificity

Since synthetic antigen was not generally available, non immune serum of the same species in which the primary antibody was raised was used, at equivalent dilutions, in TBS to check antibody specificity. Controls of this type are illustrated in Figures 2.2 and 2.3.

2.6 Reverse transcription polymerase chain reaction (RT-PCR)

2.6.1 Synthesis of oligonucleotide primers

Oligonucleotides (17-24mers) were synthesised using phosphoramadite chemistry on a model 381 DNA synthesiser (Applied Biosystems (ABI), Warrington, UK) or ordered commercially from Genosys (Pampisford, UK). Where synthesised within the laboratory, oligonucleotides were recovered into 1ml of pure ammonia, deprotected by incubating overnight at 55°C, recovered by two rounds of ethanol precipitation and resuspended in TE buffer (Appendix I). The concentration of oligonucleotide was assessed by spectrophotometry at 260nm where an optical density of 1.0 is equal to a concentration of 20μg/ml.

Figure 2.2 Control of antibody specificity. Positively-staining cells in the pars intermedia (αMSH, ACTH) and pars distalis (LHβ, PRL- prolactin) are shown on the left with equivalent controls for antibody specificity (primary antibody replaced with equivalent dilution of host-species serum) shown on the right.

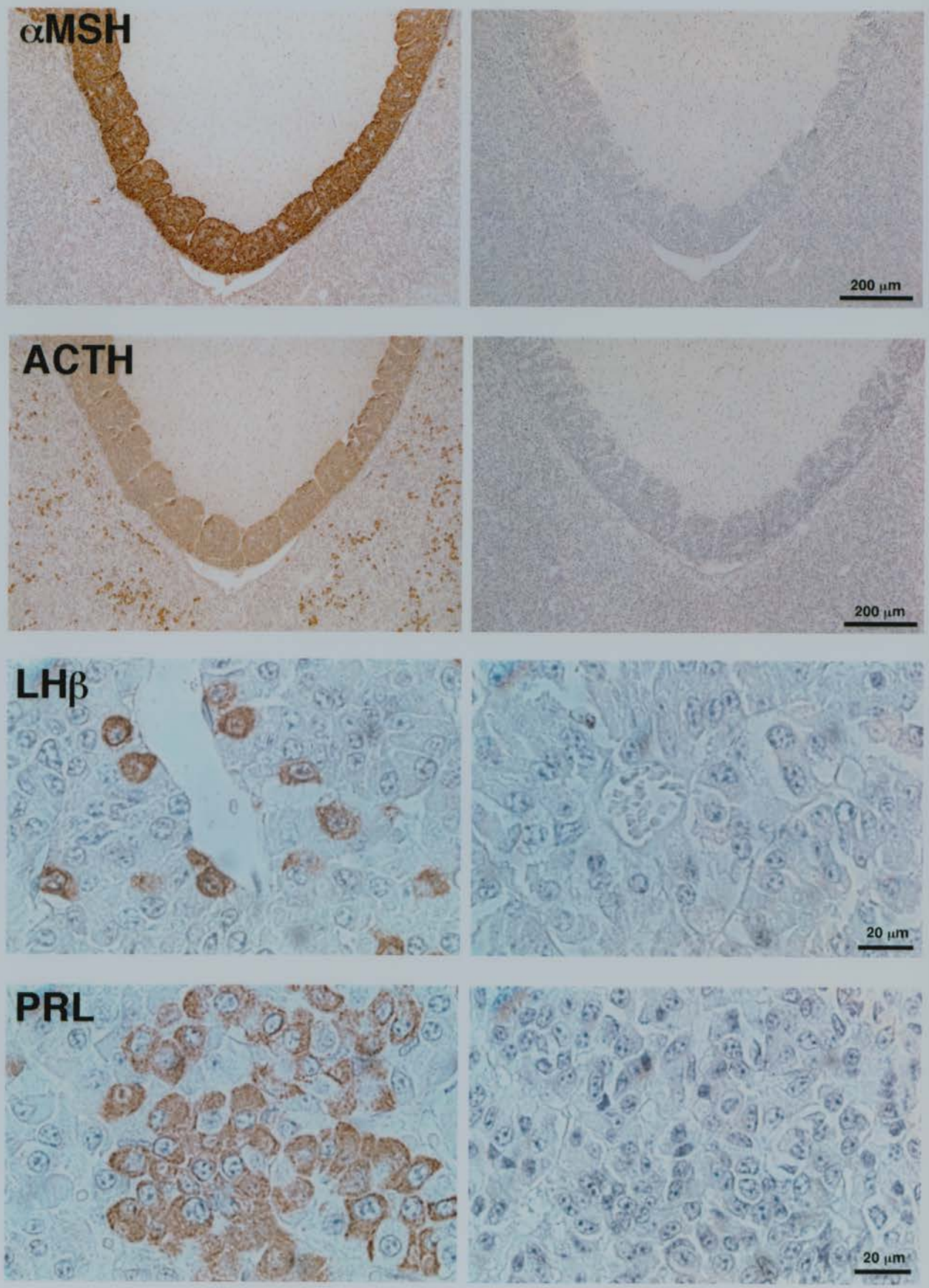
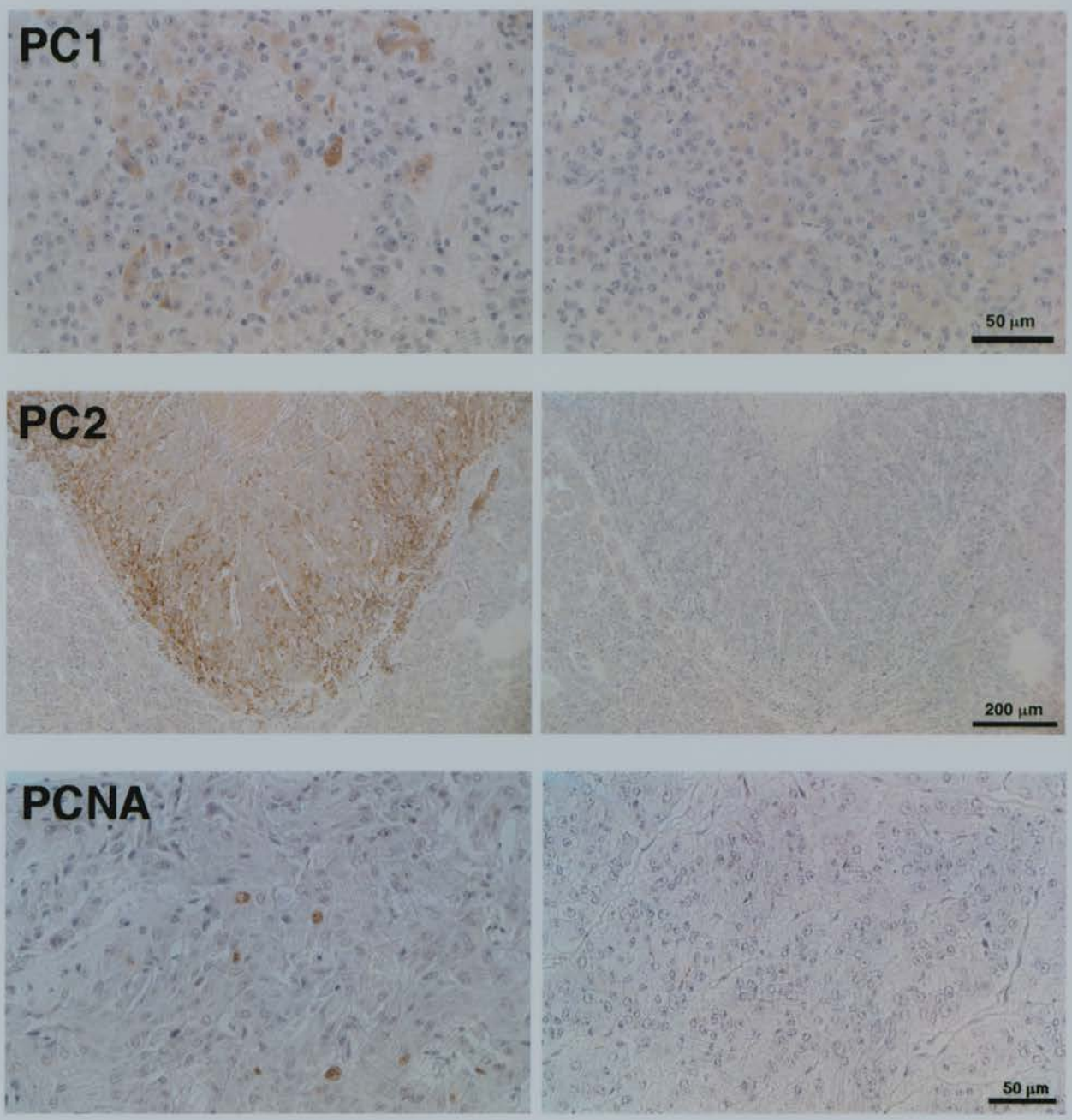

αMSH
200 μm
ACTH
200 μm
LHβ
20 μm
PRL
20 μm

Figure 2.3 Control of antibody specificity. Positively-staining cells in the pars distalis (PC1) and pars intermedia (PC2, PCNA) are shown on the left with equivalent controls for antibody specificity (primary antibody replaced with equivalent dilution of host-species serum) shown on the right.


PC1
50 μm
PC2
200 μm
PCNA
50 μm

2.6.2 RT-PCR Reaction

Partial cDNAs to ovine PC1 and PC2 mRNA were generated using an RT-PCR approach. Reverse transcription was undertaken using 250-500ng of total RNA in 20µl volume containing 10mM Tris-HCl pH 8.3, 90mM KCl, 1mM $MnCl_2$, 200µM each dNTP, 5U superscript reverse transcriptase enzyme (Gibco BRL, Paisley, UK) and 75mM of appropriate downstream 3' antisense primer or oligo dT primer (Gibco BRL) to reverse transcribe mRNA species possessing a polyT sequence at their 3' end. The conditions used for the reverse transcription were denaturation of both RNA and primer at 65°C for 5 minutes followed by immediate annealing on ice for 5 minutes. Reverse transcriptase enzyme was then added to the reaction mixture and this was incubated at 37°C for 1 hour. The reverse transcription reaction was terminated by heating to 95°C for 5 minutes. After the reverse transcription to generate the first cDNA strand was completed, PCR was undertaken on a diluted product of reverse transcription (typically 1:50) in order to amplify the products. The products of reverse transcription were diluted in a buffer containing 1.5mM $MgCl_2$, 40% glycerol, 10mM Tris-HCl pH 8.3, 890mM KCl, 6mM EGTA (to chelate $MnCl_2$ present in the reverse transcription reaction mixture) and 0.4% Tween 20. This buffer maximised the efficiency of the *Taq* DNA polymerase in the PCR reaction. 5' and 3' primers for DNA synthesis were added at a final concentration of 0.15µM.

2.6.3 Cloning of amplified cDNAs

Where RT-PCR reactions were conducted for obtaining probes for in situ hybridisation and/or RNase protection assay reactions, the amplified DNA fragments were subcloned into appropriate plasmid vectors containing SP6 and T7 RNA polymerase promoters. The vectors used were the TA cloning vectors, pCRII (Invitrogen, Abingdon, UK) illustrated in Figure 2.4 and pGEM-T easy vector, illustrated in Figure 2.5 (Promega, Southampton, UK). Ovine POMC cDNA was provided by Dr Iain Clarke (Prince Henry's Medical School, Victoria, Australia) in the bluescript pSK^- vector (Stratagene, Cambridge, UK), illustrated in Figure 2.6. Amplification of the cDNA was followed by ligation of the PCR product and plasmid at appropriate molar ratios (3:1 respectively) with T4 DNA ligase (supplied by plasmid supplier). The concentration of PCR product was assessed by comparison with known standards on an agarose gel and was added to the ligation mixture such that there was a 1:3 molar ratio of plasmid to PCR product. 25ng plasmid vector and 1U T4 DNA ligase (as supplied by plasmid supplier) were mixed with 75ng of PCR product, 1µl 10x ligation buffer (contains

60mM Tris-HCl pH 7.5, 60mM $MgCl_2$, 50mM NaCl, 1mg/ml BSA, 70mM β-mercaptoethanol, 1mM ATP, 20mM DTT and 10mM spermidine, pCRII vector or 300mM Tris-HCl pH 7.8, 100mM $MgCl_2$, 100mM DTT And 10mM ATP, pGEM-T easy vector) and made up to a volume of 10μl with water.

Following ligation, transformation of competent TOP10F' (pCRII vector) or JM109 (pGEM-T easy vector) E.Coli cells was conducted by mixing with 1μl of ligation mixture as described above. For pGEM-T easy vector, 2μl of 0.5M β-mercaptoethanol was also added to this mixture. The mixture was incubated on ice for 30 minutes, heat-shocked by incubating at 42°C for 30 seconds (pCRII) or 45 seconds (pGEM-T) and then placed on ice for 2 minutes. Thereafter 450μl SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) prewarmed to 37°C was added to the cells which were then incubated at 37°C on a shaker at 225rpm for 1 hour. Aliquots (50μl and 200μl) were plated onto Luria Bertani (LB) agar plates (Appendix I) containing 50μg/ml ampicillin, 50μl 50μg/ml X-gal and 100μl 100mM IPTG (pCRII) or 100μg/ml ampicillin, 50μl 50μg/ml X-gal and 100μl 100mM IPTG (pGEM-T) with a bent glass rod. Plates were incubated inverted overnight at 37°C. Identification of colonies transformed with plasmid containing the cDNA insert was based on the *lac Z* blue/white selection. Both pCRII and pGEM-T easy vectors contain the *lac Z* gene in their polylinker region (Figures 2.4 and 2.5). *Lac Z* gene expression was induced by IPTG to express the product β–galactosidase. This acted on the substrate X-gal, which β–galactosidase converted into a blue coloured end product. The *lac Z* gene was disrupted by the insertion of the cDNA sequence into the vector by ligation. Therefore bacterial colonies that contained plasmid without the cDNA insert had a functional *lac Z* gene and were blue whereas those colonies which were successfully transformed with recombinant plasmid were white. White bacterial colonies were restreaked on LB agar plates which were again incubated inverted overnight at 37°C. Individual bacterial colonies containing cloned plasmid were then picked into LB broth (Appendix I) and were propagated overnight at 37°C in LB broth containing 50μg/ml ampicillin in a shaker at 225 rpm. Plasmid DNA was then isolated for analysis from bacterial cultures by the alkaline lysis method using the Wizard Minipreps DNA purification system (Promega), according to the manufacturers instructions. Briefly, bacterial suspensions were pelleted by centrifugation at 1600g, the supernatant discarded, cells resuspended in buffer containing 50mM Tris/HCl, pH 7.5, 10mM EDTA and 100μg/ml RNase and mixed with an equal volume of 0.2M NaOH containing 1% SDS. The resulting solution containing lysed bacterial cells was neutralised by the addition of 2.25M potassium acetate. The resulting suspension was centrifuged at 12000g to sediment the majority of bacterial genomic DNA. The supernatant

(approximately 600µl) was removed, mixed with a 1ml suspension of DNA purification resin and passed through a miniprep column which retained the plasmid DNA bound to the resin. The plasmid DNA was then eluted into a sterile tube by the addition of 50µl pure water and subsequent centrifugation at 12000g for 20 seconds. The presence of cDNA insert of appropriate size was confirmed by restriction digestion with the endonuclease EcoRI and electrophoresis of digestion products on an agarose gel prior to definitive analysis by DNA sequencing.

2.7 Plasmid recovery and analysis

2.7.1. Plasmid preparation from bacterial cultures

Large plasmid preparations were recovered from bacterial cultures using the Wizard Maxiprep DNA purification system (Promega), according to the manufacturers instructions. Briefly, 500ml bacterial cultures were pelleted by centrifugation at 5000g, the supernatant discarded and cells resuspended in buffer containing 50mM Tris/HCl, pH 7.5, 10mM EDTA and 100µg/ml RNase. The suspension was then mixed with an equal volume of 0.2M NaOH containing 1% SDS. The resulting solution containing lysed bacterial cells was neutralised by the addition of 2.25M potassium acetate. The resulting suspension was centrifuged at 14000g to sediment the majority of bacterial genomic DNA. The supernatant (approximately 600µl) was removed and filtered. The plasmid DNA in the filtrate was then precipitated by the addition of 0.5 volumes of isopropanol and centrifuged at 14000g. The DNA pellet was then resuspended in 2ml TE buffer. This was then mixed with a 10ml suspension of DNA purification resin and passed through a maxiprep column which retained the plasmid DNA. The plasmid DNA was then eluted into a sterile tube by the addition of 1.5ml pure water and subsequent centrifugation at 14000g for 5 minutes.

2.7.2. Analysis of plasmid DNA quality

The approximate concentration of plasmid DNA was determined by scanning at 260nm on a spectrophotometer. The concentration of the DNA was calculated from the 260nm value given that an optical density of 1.0 is equal to 50µg/ml DNA.

Purity of plasmid DNA was determined by analysis on a 1.0% agarose minigel (Sambrook *et al.*, 1989). This was prepared using Seakem agarose dissolved in 0.5x TBE buffer (appendix I). The agarose was melted and approximately 200µg/ml ethidium bromide was added for visualisation of DNA.

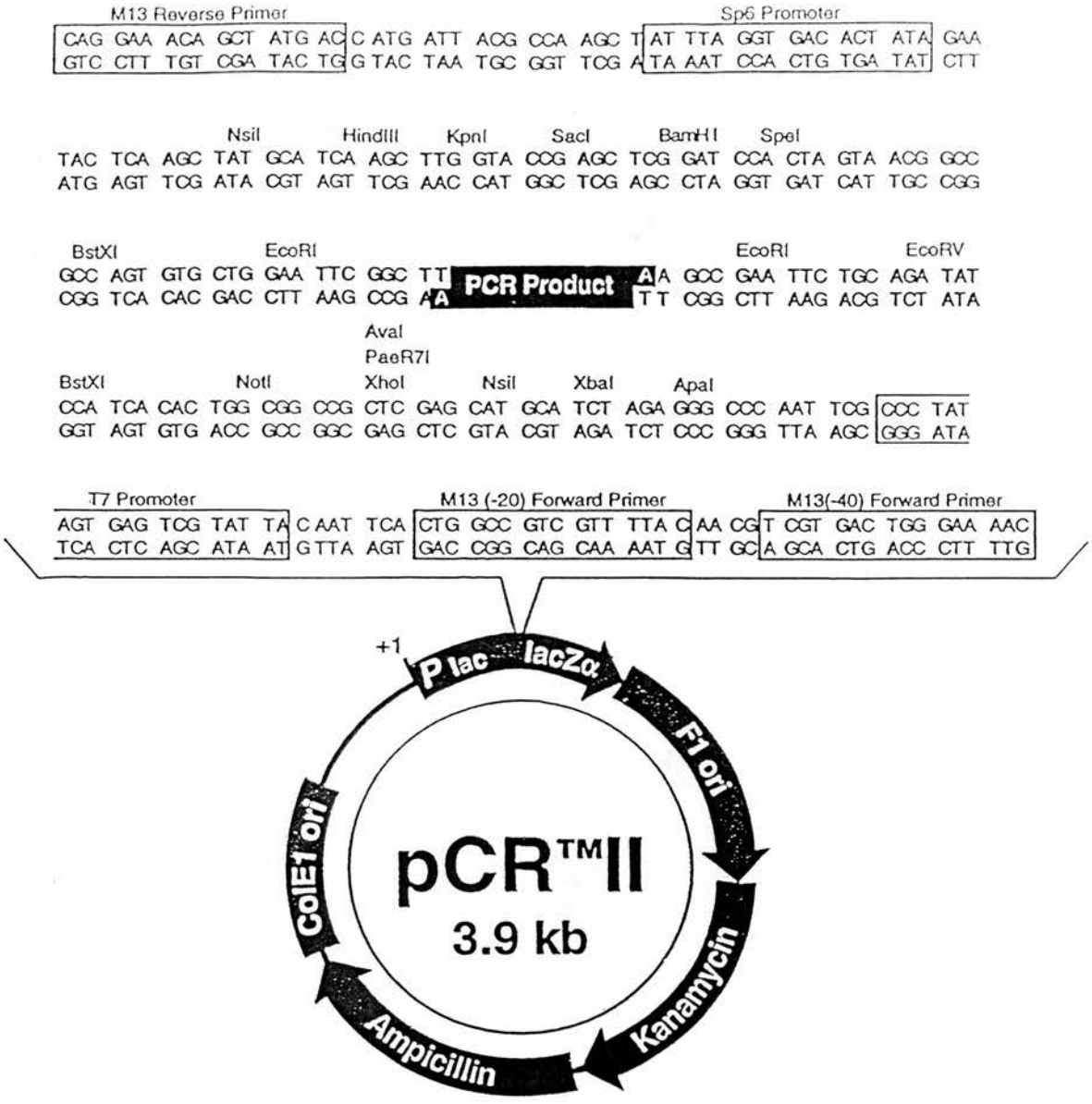


Figure 2.4 pCRII TA cloning vector. *Adapted from Invitrogen product catalogue.*

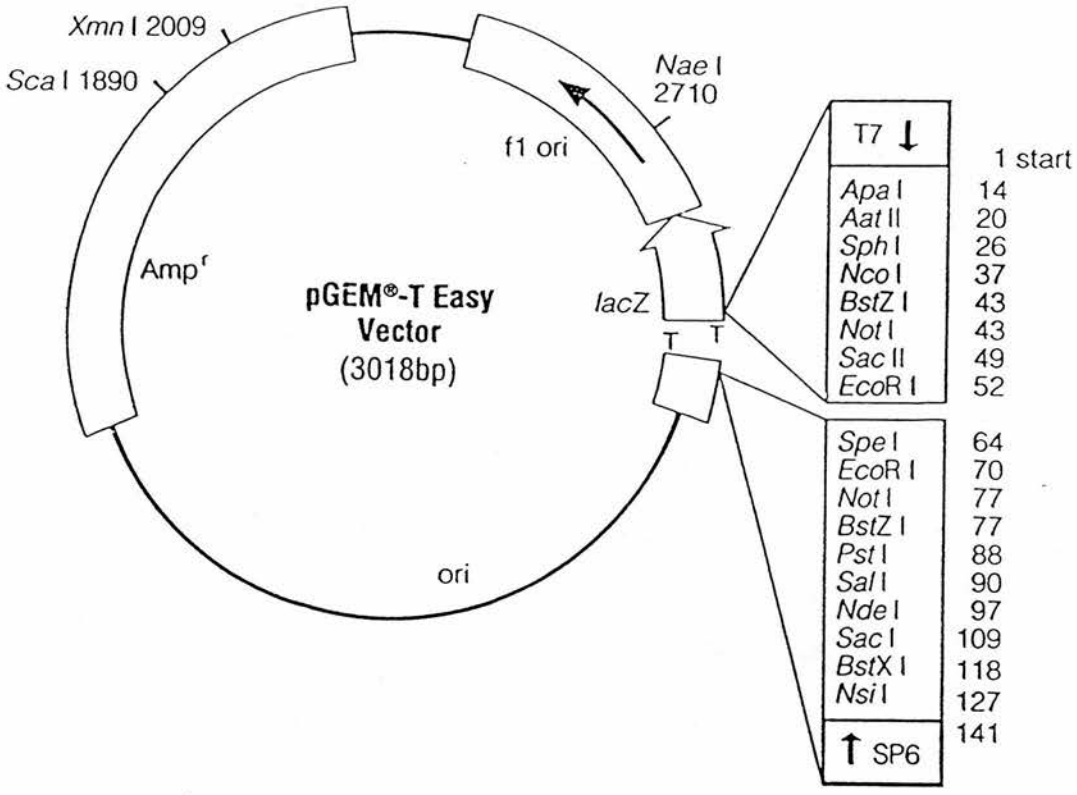


Figure 2.5 pGEM-T Easy vector. *Adapted from Promega product catalogue.*

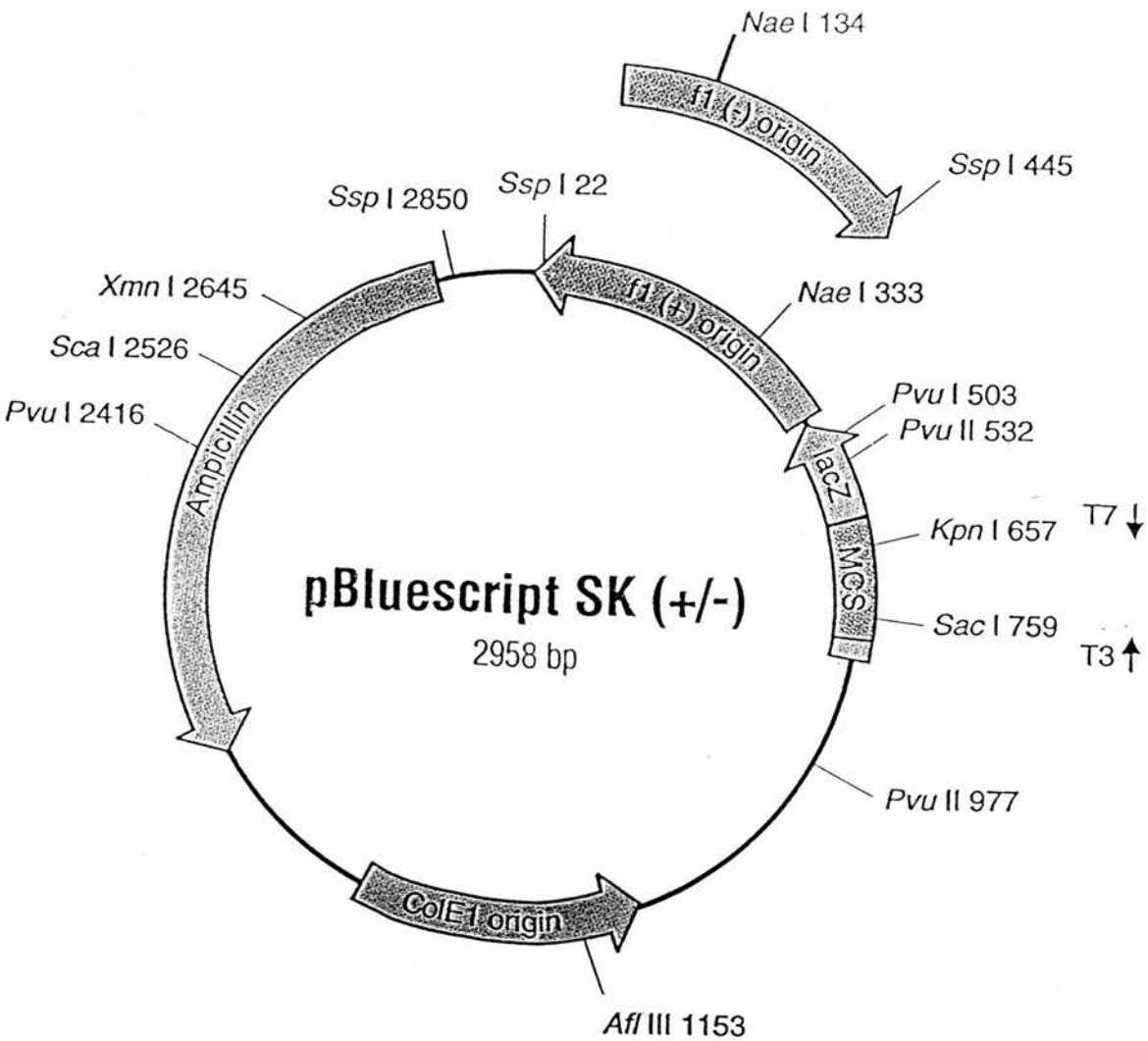


Figure 2.6 Bluescript SK$^-$ vector. *Adapted from Stratagene product catalogue.*

The gel was poured into a 7cm by 10cm gel tray containing an 8 well comb and submerged in 0.5 x TBE buffer in a midigel cell (Hybaid). Plasmid DNA (10µl) was run in a sample solution containing 2µl loading buffer (contained 30% w/v glycerol, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF and 0.5M EDTA at pH7.0) and 8µl water. Samples were separated in parallel with KB Ladder DNA markers (range 72-23130bp, Gibco BRL) by electrophoresis at 100V for 1-2 hours in 0.5x TBE, viewed under UV light and photographed.

2.8. DNA sequencing

2.8.1. Automatic sequencing

Automatic sequencing reactions were carried out using the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and were run on the Applied Biosystems Model 373A DNA sequencing system.

2.8.1.1. Sequencing reactions

A reaction premix was prepared containing 4µl 5x TACS buffer (400mM Tris/HCl, 10mM $MgCl_2$ and 100mM $(NH_4)_2SO_4$ pH9.0), 1µl dNTP mix (containing 150µM each dATP, dGTP, dTTP and dCTP), 1µl each ddATP, ddTTP, ddCTP and ddGTP terminators and 0.5-1µl AmpliTaq DNA polymerase for each sequencing reaction to be performed. A total of 11.5µl (PCR template) or 9.5µl (plasmid DNA template) of the reaction premix was added to separate tubes containing 3.2 pmol primer and 5µl template DNA, the total volume adjusted to 20µl with water and overlaid with mineral oil.

Tubes were placed in a Perkin Elmer Cetus Model 480 thermal cycler preheated to 96°C and 25 cycles of thermal cycling carried out as follows; rapid thermal ramp to 96°C, 96°C for 30 seconds, rapid thermal ramp to 50°C, 50°C for 15 seconds, rapid thermal ramp to 60°C and 60°C for 4 minutes.

On completion of the sequencing reaction, the samples were extracted twice with water saturated phenol:chloroform and precipitated with 15µl 2M sodium acetate pH4.5 and 300µl cold ethanol to remove unincorporated labelled terminators. Labelled DNA was pelleted by centrifugation, the supernatant discarded and the DNA washed using 70% ethanol, air dried and resuspended in formamide/EDTA buffer (5µl formamide, 1µl 50mM EDTA, pH8.0) just before loading onto the gel.

2.8.3.2. Automatic sequencing gel

A sequencing gel mix containing 50g urea, 15ml 40% acrylamide and water made up to a final volume of 80ml was prepared. Amberlite resin (Sigma) was added to remove acrylamide free acid and the mixture was stirred and heated until the urea had dissolved, the solution was filtered and 10ml 10x TBE added. The gel mix was polymerised using 45µl TEMED and 500µl 10% ammonium persulphate and poured carefully between clean glass plates.

The gel was prerun for 30 minutes at 30W. Samples resuspended in buffer were heated to 95°C for 5 minutes before loading onto the gel. The gel was run overnight using the automatic data collection and analysis programs. The following cDNA probes, sequenced in this way, were used in the studies described in this thesis.

2.8.2. Ovine POMC cDNA probe

The sequence of the ovine POMC cDNA probe (provided by Dr Iain Clarke) used in this study is shown in Figure 2.7. This sequence codes for a portion of the POMC peptide at the C-terminal, including βEND, and continues into the 3' untranslated region.

2.8.3 Ovine PC1 probe

The novel ovine PC1 cDNA probe used in this study was generated by an RT-PCR approach using primers GAGATAACTGTGACTGTGAT (5') and TCGGCTGTTCACCATCAAGCC (3'-reverse), based on nucleotide sequences occurring at 1162 and 1514bp of the rat PC1 cDNA sequence (accession number M76705, (Bloomquist *et al.*, 1991)). A 374bp fragment was generated with 88% homology to the rat and 95% homology to the porcine (accession number U20545, (Dai *et al.*, 1995)) nucleotide sequences between the primer sequences (Figure 2.6). The predicted ovine peptide sequence was identical to the predicted rat peptide. The ovine PC1 nucleotide sequence had less than 50% homology with published PC2 or any other endoprotease sequences. This cDNA was subcloned into the pCRII vector, sequenced and deposited on the NIH genbank database, accession number AF063110.

2.8.4 Ovine PC2 probe

The novel ovine PC2 cDNA probe used in this study was generated by an RT-PCR approach using primers GATGACTGGTTCAACAGCCAT (5') and GCCGTCACAGTTGCAGTCATC (3'-reverse), based on nucleotide sequences occurring at 923 and 1262bp of the rat PC2 cDNA sequence (accession number M76706, (Bloomquist *et al.*, 1991). A 360bp fragment was generated with 88% homology to the rat and 91% homology to the porcine (accession number X68603,

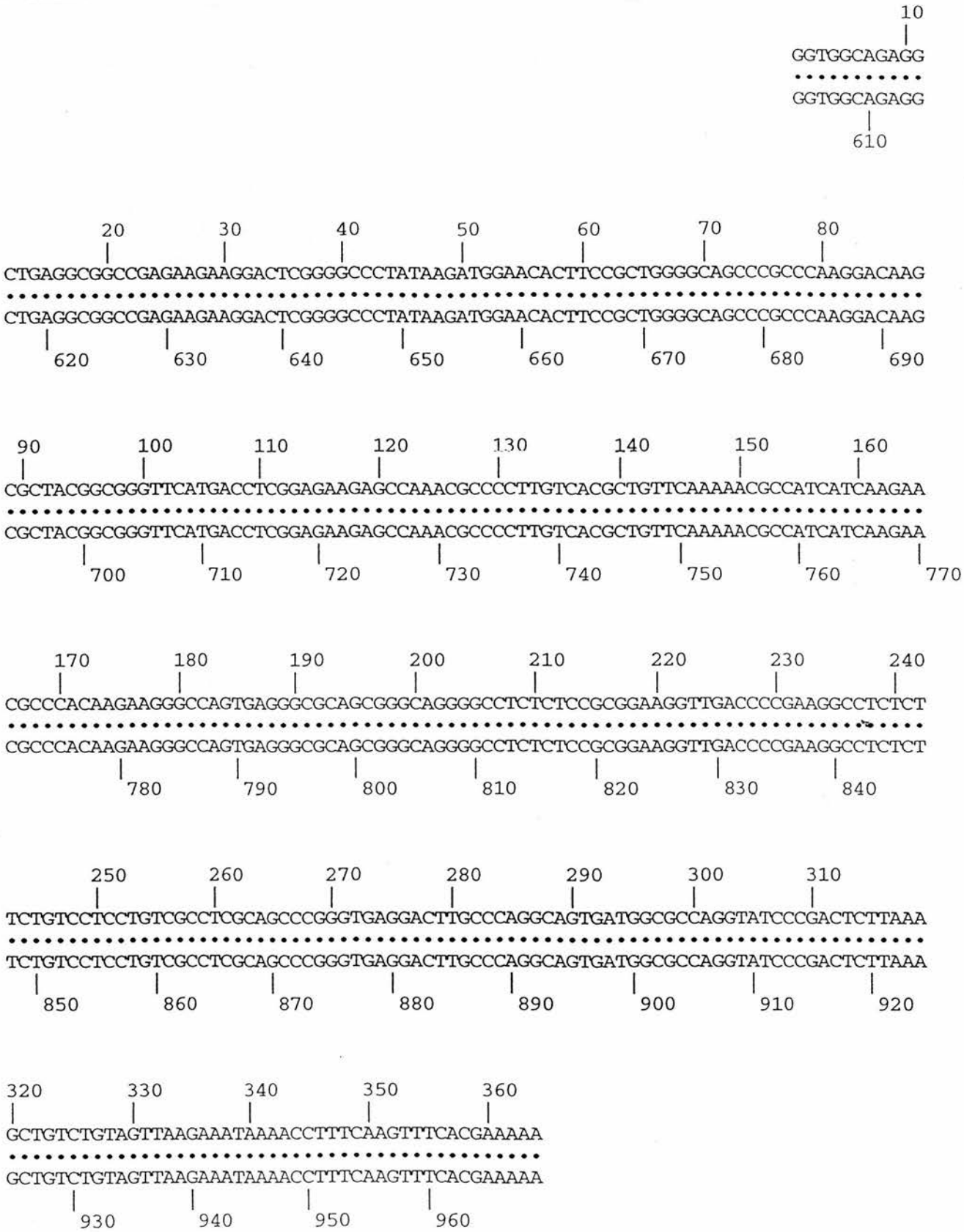


Figure 2.7 POMC cDNA probe sequence. The cDNA used in this study is shown (top) with part of the full ovine POMC cDNA (bottom). Numbers refer to bases from the 5' end of the probe (top) or from the start of translation site in the full sequence (bottom).

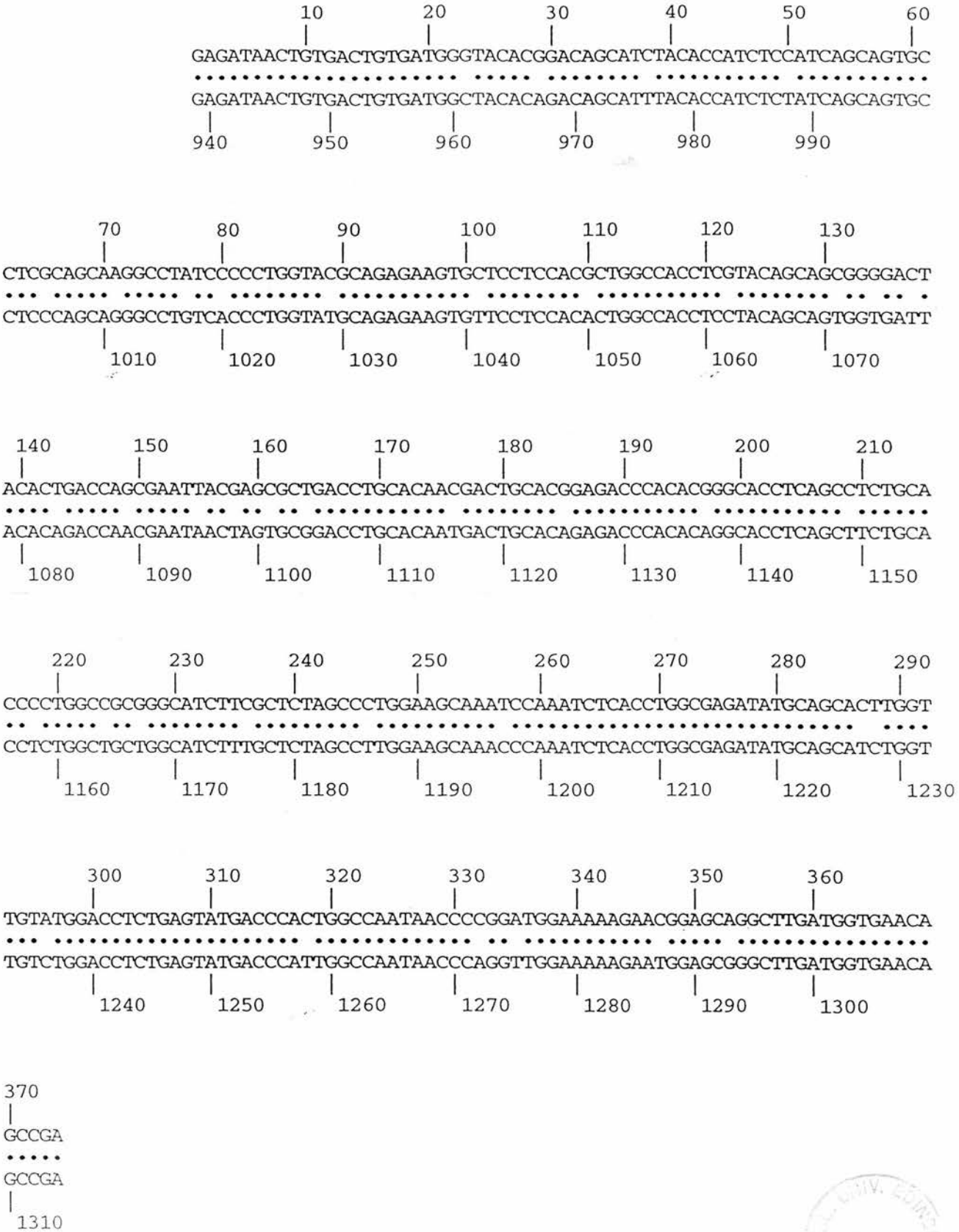


Figure 2.8 PC1 cDNA probe sequence. The cDNA used in this study is shown (top) with part of the rat PC1 cDNA (bottom). Numbers refer to bases from the 5' end (ovine cDNA) or from the start of translation site (rat sequence). Rat primer sequence is underlined.

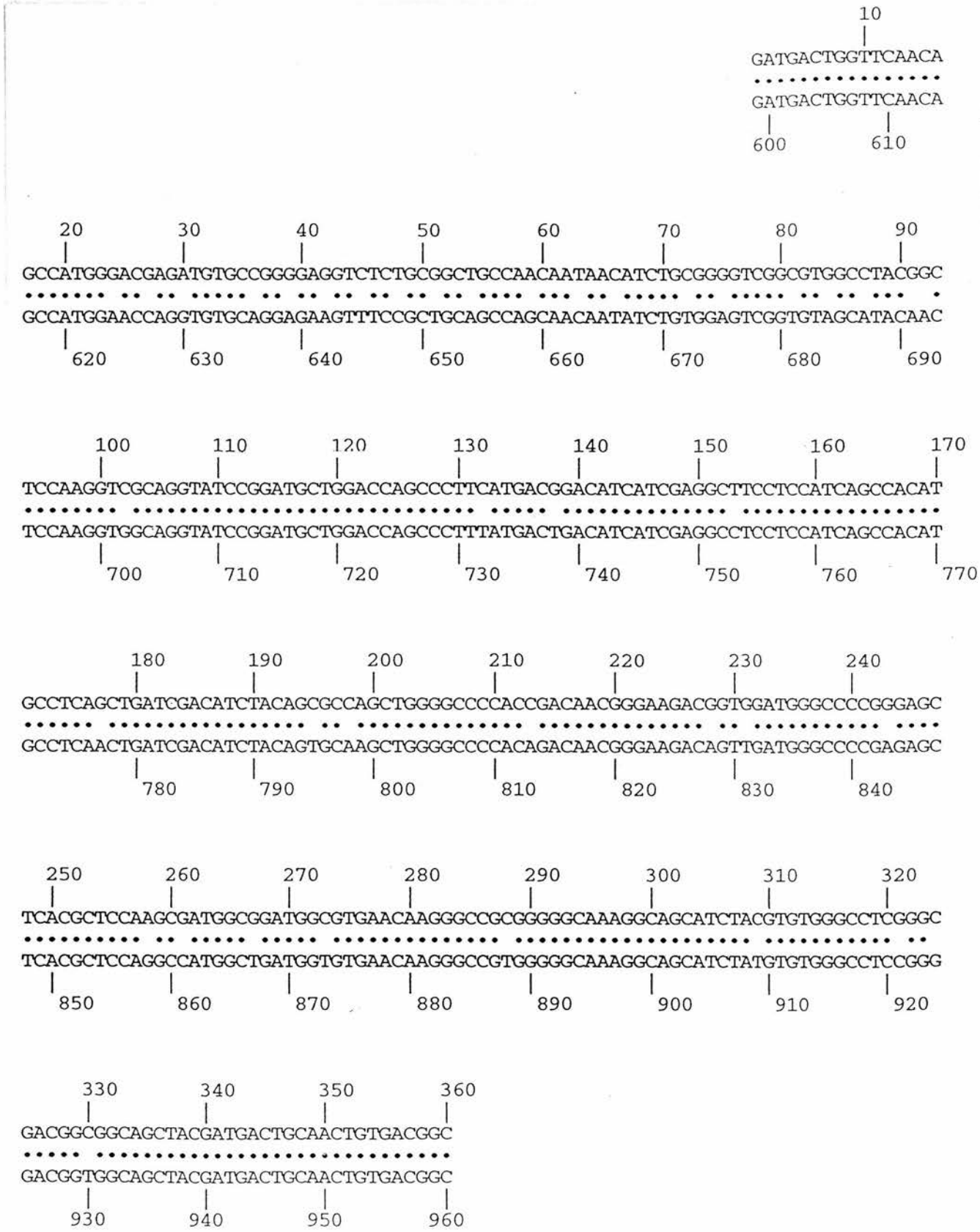


Figure 2.9 PC2 cDNA probe sequence. The cDNA used in this study is shown (top) with part of the rat PC2 cDNA (bottom). Numbers refer to bases from the 5' end (ovine cDNA) or from the start of translation site (rat sequence). Rat primer sequence is underlined.

(Seidah *et al.*, 1992) sequence between the primer sequences (Figure 2.7). The predicted peptide sequence was 98% homologous to the predicted rat peptide. The nucleotide sequence had less than 50% homology with published PC1 sequences, although it shared approximately 60% homology to the human PACE4 and rat PC5 sequences (Johnson *et al.*, 1994; Seidah and Chretien, 1994). This level of homology is unlikely to interfere with any of the detection techniques subsequently used given that hybridisation and post-hybridisation conditions were kept relatively stringent. This cDNA was subcloned into the pGEM-T vector. The nucleotide sequence was deposited on the NIH genbank database, accession number AF063108.

2.9. Preparation of radiolabelled probes for Northern blot analysis

2.9.1. Preparation of template DNA for radiolabelling

Double stranded DNA for radiolabelling was prepared by excision of the cloned cDNA insert from the plasmid vector by enzymatic digestion and separation on a 1.0% low melt agarose (Seakem, UK) gel. The appropriately sized DNA fragment was cut out of the gel, melted at 65°C and this was diluted 1:5 in pure water to prevent re-solidification. The presence of agarose at this concentration did not appear to interfere with the random labelling procedure. The quantity of excised cDNA was estimated by comparison with a known concentration of KB Ladder.

2.9.2. Radiolabelling double stranded DNA

Double stranded cDNA was used to produce radiolabelled cDNA using the random primer method (Feinberg and Vogelstein, 1983) and an Amersham 'Rediprime' kit according to the manufacturers instructions (Amersham). Briefly, DNA (2.5-25ng) was denatured at 98°C for 5 minutes. DNA was labelled with 50µCi of ^{32}P-[α]-dCTP in a reaction containing 5µl primer solution of random hexanucleotides, 10mM each dATP, dGTP and dTTP, and 5µl reaction buffer containing Tris/HCl pH7.8, $MgCl_2$ and 2-mercaptoethanol. The labelling reaction was catalysed by addition of 2U Klenow enzyme and incubation was for 30 minutes at 37°C. Labelled DNA was denatured with 5N NaOH (100µl), neutralised with 1M Tris, pH7.6 (600µl) and 1N HCl (375µl) and added to the hybridisation mixture (section 3.7).

The generation of high specific activity cDNA probes was confirmed by pipetting 1µl of the newly synthesised probe on glass fibre filter paper (Whatman

GF, Whatman, Maidstone, UK). This was washed for three times two minutes in 5% Trichloroacetic acid (TCA) then in 70% ethanol and finally rinsed with 95% ethanol and air dried before analysis in a beta counter.

2.10. Northern blot analysis

2.10.1. Separation of RNA on denaturing agarose gels

RNA was separated on 1.0% denaturing agarose gels both for assessment of RNA quality and prior to blotting onto nitrocellulose membranes for northern analysis (section 2.10). The gel was prepared by melting 1.1g Seakem agarose (FMC supplied by Flowgen, Sittingbourne, UK) in 70ml DEPC-treated water. This was cooled to about 60°C and 20ml of 10x northern running buffer (containing 200mM MOPS, 10mM EDTA and 50mM sodium acetate at pH7.0) plus 22ml 37% formaldehyde were added. The solution was mixed gently and poured into a gel tray (15 x 20cm) containing a 12 well comb in a fume hood. After setting, the comb was removed and the gel submerged in 1x northern running buffer in a midigel electrophoresis cell (Hybaid, Ashford, UK).

RNA (5µg) in a volume of no greater than 4.5µl was prepared by adding 2.0µl 10x MOPS northern-running buffer (Appendix I), 4.5µl of formaldehyde and 10µl deionised formamide and heating at 60°C for 5 minutes. After heating, 2µl of northern-loading buffer (contains 50% w/v glycerol, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol and 0.5M EDTA at pH7.0) and 1µl of 1mg/ml ethidium bromide was added to each sample. RNA was loaded into individual wells of the gel and separated by running for approximately 6 hours at 60V. An RNA ladder (Gibco BRL) was run with the RNA so that RNA transcript size could be accessed.

2.10.2. RNA transfer to membrane

RNA separated on a denaturing gel was transferred to nylon membrane (Hybond-N; Amersham) by capillary blotting as shown in Figure 2.10. The nylon membrane was prewetted with pure RNase-free water followed by 20x SSC before placing carefully on the gel. Transfer using 20x SSC proceeded for a minimum of 12 hours. After transfer the position of the gel wells were marked on the membrane and RNA was bound by UV crosslinkage.

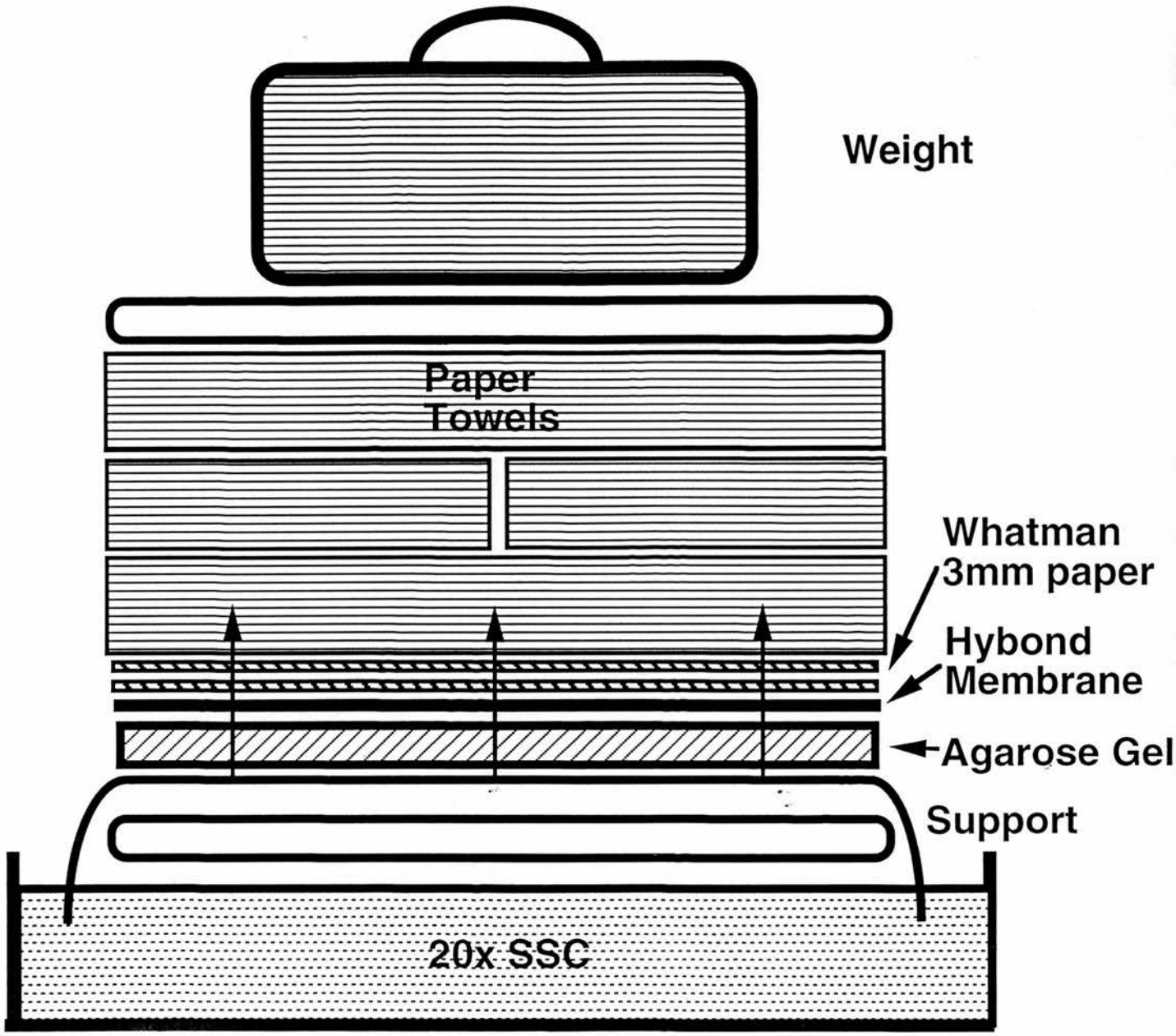


Figure 2.10 Northern blotting. Schematic drawing of the apparatus used for the transfer of RNA from a denaturing agarose gel to Hybond-N nylon membrane (black) by capillary action.

2.10.3. Hybridisation of radiolabelled probe to membrane

Membranes were prehybridised at 65°C for 2-4 hours in buffer containing 20% formamide, 5x Denhardt's solution (Appendix I), 5x SSPE buffer (Appendix I), 0.1% SDS, 0.1mg/ml denatured salmon sperm DNA (Sigma), 1µg/ml polyA (Sigma) and 6% dextran sulphate (Sigma). Radiolabelled probe was added to the prehybridisation mix to give a final activity of 0.5 - $1x10^6$ cpm/ml buffer. Hybridisation was allowed to proceed for 24-48 hours at 65°C.

2.10.4. Post-hybridisation washes

Membranes were washed with 2x SSC, then 1x SSC, 0.5x SSC or 0.2x SSC for 15 minutes each at 55°C.

2.10.5. Development of signal

After washing, membranes were wrapped in clingfilm and exposed to X-ray film (X-OMAT AR or LS; Kodak, supplied by Sigma) in cassettes at -70°C. After an appropriate exposure time the signal was developed using LX 24 developer (Kodak) and fixed using FX 40 fixative (Kodak) according to the suppliers recommendations.

2.11. Radioactive *in situ* hybridisation

2.11.1 Preparation of radiolabelled cRNA probes.

2.11.1.1. Template cDNA preparation

Plasmid DNA (1µg) prepared as described previously in section 3.7 was linearised in reaction buffer (contains 10-50mmol/l Tris/HCl, 5-10 mmol/l $MgCl_2$, 50-100mmol/l NaCl and 1mmol/l DTT, DTE or ß-mercaptoethanol) with 10U restriction enzyme and made up to a volume of 10µl with pure water. The enzyme and buffer used was dependent on the plasmid vector being digested and the direction of synthesis of the riboprobe. These are given in Table 2.1. The reaction was incubated at 37°C for 2-4 hours. Digested DNA was then extracted once with tris-buffered phenol:chloroform, once with chloroform alone and then precipitated with 0.1 volumes 3M sodium acetate (pH5.5) and 2.5 volumes absolute ethanol overnight at -20°C. Linearised DNA was pelleted, dried and resuspended in 9µl pure water. Thereafter, 1µl of reaction mix was run on a minigel (1.0% agarose)

and compared to an uncut sample of the same plasmid run in a parallel lane to assess the efficiency of digestion.

Table 2.1. Restriction Endonucleases used to linearise plasmids for use as riboprobe synthesis templates and RNA polymerases used to synthesise probes. AS, Antisense, S, Sense. *18S for use in RNase protection assay as supplied prelinearised by Ambion (Austin, TX, USA).

cDNA	Plasmid	Orientation	Endonuclease
POMC	Bluescript	AS (T7)	BamHI
POMC	Bluescript	S (T3)	Hind III
PC1	pCRII	AS (SP6)	Xba I
PC1	pCRII	S (T7)	BamHI
PC2	pGEM-T	AS (T7)	Nde I
PC2	pGEM-T	S (SP6)	Nco I
18S*	pTRI	AS (T7)	-

2.11.2. In vitro synthesis of cRNA probes

Synthesis of riboprobes was carried out using 1μg linearised plasmid template in a reaction mix containing 4mM DTT, 40U RNase inhibitor, 0.4mM each rATP, rCTP and rGTP, 8μM rUTP, 1x transcription buffer (1x transcription buffer contains 40mM Tris/HCl, pH7.9, 6mM $MgCl_2$, 2mM spermidine and 10mM NaCl) and 50μCi ^{33}P-UTP. The reaction was catalysed by addition of 20U of the appropriate RNA polymerase (T3, T7 or SP6) and incubated at 37°C for approximately 1.5 hours. The DNA template was then removed by digestion with 10U RNase-free DNase for 15 minutes at 37°C. 20μg of tRNA (from bakers yeast, Sigma) was added to assist in precipitation pellet formation. Enzymes and salts were removed by phenol/chloroform and chloroform extractions and RNA was precipitated with sodium acetate and ethanol for at least 1 hour at -70°C and recovered by centrifugation as above.

Radiolabelled RNA was air dried, resuspended in 50μl pure water and the activity of 2x 1μl aliquots was determined by liquid scintillation spectroscopy. The average activity of the two samples was calculated and the volume of probe necessary to give 1 x 10^6 cpm was determined.

2.11.3. Pretreatment of tissue

Paraffin wax embedded tissue was cleared in histoclear for 10 minutes and rehydrated in a series of alcohols of decreasing concentrations. Tissue was placed in 0.2N HCl for 20 minutes followed by two 5 minutes washes in distilled water.

Sections were then treated with 2μg/ml proteinase K (Sigma) in buffer containing 100mM Tris/HCl pH7.8 and 50mM EDTA at 37°C for 20 minutes followed by distilled water for 2 minutes. Sections were washed briefly in 0.1M triethanolamine (TEA) pH8.0 and acetylated in 0.25% acetic anhydride in 0.1M TEA pH8.0 for 10 minutes. Finally, sections were rinsed in distilled water and prehybridised in buffer containing 4 x STE buffer (Appendix I), 1 x Denhardt's solution, 10mM DTT, 125μg/ml salmon sperm DNA, 125μg/ml yeast transfer RNA in 50% deionised formamide for 2-4 hours at probe Tm-25°C.

2.11.4. Hybridisation of probe to tissue

Hybridisation was conducted overnight using hybridisation buffer (as prehybridisation buffer with 10% dextran sulphate) containing radiolabelled probe at 1 x 10^6 cpm in 40μl buffer/slide. The incubation was carried out under coverslips prepared from Gelbond film (Flowgen), hydrophilic side towards the tissue, in a humidified chamber at Tm-25°C where Tm was calculated individually for each probe according to the equation:

$$Tm = 79.8 + 58.4\ (F_{GC}) + 11.8\ (F_{GC})^2 + 18.5 \log(M) - 820/L - 0.35\ (\%F) - (\%M)$$

where:

F_{GC} = mole fraction of GC content of probe, usually about 0.45

M = monovalent cation concentration (molarity of salt in hybridisation buffer)

L = length of duplexes formed during hybridisation (probe length)

%F = percentage of formamide in buffer

2.11.5. Post hybridisation washes

After incubation sections were washed in two changes of 4x SSC for 10 minutes each to remove the coverslip and treated with RNase A (Sigma) at a concentration of 20μg/ml in 0.5M NaCl, 10mM Tris pH8.0 and 1mM EDTA for 30 minutes at 37°C. Sections were washed in RNase buffer alone for 30 minutes at 37°C followed by 30 minute washes in 4x SSC and 2x SSC at room temperature and a final wash in 0.1x SSC at 55°C for 30 minutes. Sections were then dehydrated in alcohols of increasing concentration all containing 300mM ammonium acetate.

2.11.6. Development of *in situ* hybridisation

After air drying, slides were warmed to 45°C and dipped in prewarmed NTB3 photographic emulsion (Kodak) at 45°C in the dark. Emulsion coated slides were stored in a humidified, lightproof box overnight before transfer to a lightproof

polyacetyl black trough (Lamb's laboratory supplies, London, UK) containing silica gel and stored at 4°C for 1-2 days (POMC) or six weeks (PC1 and PC2).

Silver grains formed by reaction of the hybridised, radiolabelled probe with the emulsion were developed using Kodak D19 developer. Briefly, in the dark, slides were placed in developer cooled to 15°C for 4 minutes, followed by a 20 second wash in pure water, then fixation by incubation for 10 minutes in Kodak polymax at 14°C. Sections were washed in pure water for 20 minutes, rinsed in running tap water for 20 minutes, stained with haematoxylin, dehydrated and mounted in Pertex (O.Kindler GmbH & Co. supplied by Laboratory Sales Ltd., Rochdale, England).

Slides were analysed under dark field using an Olympus BH2 microscope (Leitz, Wetzlar, Germany) to visualise the silver grains indicating areas of hybridisation. Silver grain density was analysed using image analysis software (Image proplus, Media Cybernetics, Silver Springs, MD, USA). Bright field microscopy was used to examine the morphology of the pituitary gland sections and to allow identification of the pars intermedia, nervosa and distalis.

2.12 Ribonuclease (RNase) protection assay.

2.12.1 Preparation of radiolabelled cRNA probes

Radiolabelled antisense cRNA probes were prepared as described in section 2.11 except that ^{32}P-[α]-UTP was used in place of ^{33}P-UTP and no cold rUTP was added to the in vitro transcription reaction in the preparation of PC1 and PC2 antisense cRNA probes. As a control for sample RNA loading, 18S antisense RNA probe (Ambion, supplied by AMS Biotechnology (UK), Witney, UK) was prepared according to the manufacturers instructions. In order to purify full length cRNA transcripts, radiolabelled RNA probes were gel-purified. RNA probes were mixed with gel loading buffer II (Ambion, contains 95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5mM EDTA, 0.025% SDS), heated at 95°C for 3 minutes and loaded onto a 6% denaturing polyacrylamide gel and separated by electrophoresis at 300V for 2 hours. After electrophoresis, the gel was wrapped in cling film and exposed briefly (1-2 minutes) to an autoradiographic film (X-OMAT AR, Kodak). The portion of the gel containing the full length transcript was excised and the probe was eluted in elution buffer (Ambion, contains 0.5M ammonium acetate, 1mM EDTA, 0.025% SDS) at 37°C overnight. The activity of two 1μl aliquots of the probe in elution buffer was then measured by liquid scintillation spectroscopy and from the average of these the volume of probe having an activity of 2x 10^5 cpm was determined (2x 10^4 for 18S control probe).

2.12.2 RNase protection assay reaction

Reagents used in RNase protection assay were supplied by Ambion. Total RNA extracted as described in section 3.3 was mixed with $2x10^5$ cpm of radiolabelled cRNA and $5x10^4$ cpm of 18S antisense probe. These were coprecipitated with 0.1 volumes 5M ammonium acetate and 2.5 volumes absolute ethanol, resuspended, denatured at 95°C for 3 minutes and hybridised overnight in 20ml hybridisation buffer (contains 80% deionised formamide, 100mM sodium citrate pH 6.4, 300mM sodium acetate pH 6.4 and 1mM EDTA) at 44°C. After hybridisation of probes to RNA, non-hybridised single-stranded RNA was selectively digested by the addition of 5U RNase A and 200U RNase T1 (Ambion) in RNase buffer at 37°C for 40 minutes. The reactions were then precipitated in the presence of 0.3M sodium acetate and absolute ethanol for 30 minutes at -20°C and centrifuged at 13000g for 20 minutes at 4°C. The supernatant was carefully removed and the pellet redissolved in 8µl gel loading buffer, heated to 95°C for 3 minutes and loaded onto a 6% denaturing acrylamide gel prepared as described below. Undigested and unprotected controls were included to check for full RNase digestion. The sizes of the protected fragments was accessed by running radiolabelled RNA size standards on the gel. These were prepared from prelinearised plasmids by in vitro transcription according to the manufacturers instructions (RNA Century Marker Plus template set, Ambion). Samples were run for 2 to 3 hours at 200V before drying the gel at 80°C and exposing to either an autoradiographic film (Kodak) or a phosphorescent screen for quantification with a phosphorimager (Storm, Molecular Dynamics, Sunnyvale, CA, USA). The relative optical density of the cDNA probe-protected and 18S ribosomal standard were determined on a phosphorimager and the 18S ribosomal standard was used to correct for loading variations in the RNA between treatments.

2.12.3 Denaturing acrylamide RNase protection assay gel

A gel mix containing 14.4g urea, 3.8ml 40% acrylamide (19:1 acrylamide: bisacrylamide) and 3ml 10x TBE made up to a final volume of 30ml was prepared and the mixture was stirred and heated until the urea had dissolved. The gel mix was polymerised using 32µl TEMED and 240µl fresh 10% ammonium persulphate and before complete polymerisation was poured carefully between clean glass plates. A comb was inserted to form wells for sample loading. The gel was run in 1x TBE buffer.

2.13 Radioimmunoassay

In selected experiments, circulating concentrations of αMSH, prolactin and FSH were determined in single radioimmunoassays using methods validated for sheep plasma (αMSH, (Lincoln and Baker, 1995); prolactin, (McNeilly and Andrews, 1974); and FSH, (McNeilly *et al.*, 1986)). For the αMSH assay, the αMSH antibody used crossreacted at 0.15% with ovine ACTH and 70%with ovine desacetylated αMSH. The lower limit of detection (10% decrease in binding relative to B_0) was 22.5 pmol/litre and the intra-assay coefficient of variation was 16%. The corresponding values for the prolactin assay were 0.5 μg NIH prolactin S13/litre and 6.0%. For the FSH assay these were 0.2 μg/NIDDK-FSH-RP2/litre and 9.3%

2.14 Statistical analyses

Differences between the relative density in the different treatment groups were evaluated using ANOVA. Comparisons between individual groups were carried out using the unpaired student t-test. Where standard deviations differed between treatments, the natural logarithms of data were used for parametric comparisons. Results both in histograms and in the text are presented as mean±the standard error of the mean (SEM).

Chapter 3

The anatomy of the Soay ram pituitary gland

3.1 Introduction

The general anatomy of the sheep pituitary gland is well described. The pituitary gland is divided into anterior and posterior lobes with a clearly defined pars intermedia. The pars intermedia develops in fetal life and remains a major part of the pituitary gland throughout adult life. The tissue is closely associated with the pars nervosa and forms a layer of approximately 15-20 cells thick between the pars nervosa and pars distalis. The ovine pars intermedia is a lobular tissue with a cell population predominantly composed of a single endocrine cell type, the melanotroph (Perry *et al.*, 1981). POMC gene expression in the pars intermedia is detected by Day 70 of fetal life and is the predominant gene expressed in the melanotrophs in the adult. POMC is also expressed by the corticotrophs dispersed throughout the pars distalis (Castro and Morrison, 1997; Smith and Funder, 1988).

The aim of this study was to characterise the anatomy of the Soay sheep pituitary gland using immunocytochemistry. In particular, the localisation of the POMC products αMSH and ACTH and the localisation of the associated endoproteolytic POMC-processing enzymes PC1 and PC2. The prediction was that cell types expressing αMSH would be characterised by the expression of both PC1 and PC2 while the cells expressing predominantly ACTH would express PC1 alone. Additionally, the distribution of two other cell types, gonadotrophs and lactotrophs was characterised as markers of the pars distalis (Polkowska *et al.*, 1980; Skinner and Robinson, 1995).

3.2 Experimental procedures

3.2.1 Animals and collection of tissues

Pituitary glands were collected from adult Soay rams maintained indoors under mixed artificial photoperiodic conditions as described in section 2.1.2. Animals were housed in individual pens and fed on a constant diet of dried grass nuts with hay and water *ad libitum*. Animals were killed and immediately after death, the pituitary gland was removed, weighed and promptly fixed for five hours in Bouins reagent and processed for histology as described in section 2.4.

3.2.2 Immunocytochemistry

Immunocytochemical staining for αMSH, ACTH, PC1, PC2, LHβ and prolactin was carried out on 3μm coronal sections of the pituitary gland (Section 2.5.4). A polyclonal rabbit antibody against ovine CLIP was used for the detection of ACTH. Anti-C-terminal ovine αMSH rabbit polyclonal antibody was used to identify the melanotrophs and rabbit polyclonal anti-ovine LHβ antibody to identify the gonadotrophs. Rabbit polyclonal anti-ovine prolactin antibody was used to identify the lactotrophs. Rabbit polyclonal anti-mouse PC1 and rabbit polyclonal anti-mouse PC2 antibodies were used to localise the site of expression of the processing enzymes. Immunocytochemistry utilised the avidin-biotin horse radish peroxidase complex (Section 2.5.2). Where double immunocytochemistry was employed both the horse radish peroxidase and the alkaline phosphatase anti-alkaline phosphatase methods were employed simultaneously (Section 2.5.3). Control sections were incubated with non-immune serum from the species in which the respective primary antibody was raised (Section 2.5.5).

The relative size of the different compartments of the pituitary gland were measured in cross section using an image analysis program (Proplus software). Cell frequency as a percentage of total cells within a tissue was calculated by counting total and positively-staining cells within ten graticule units (Graticules Ltd, Tonbridge, UK) in ten different areas of the tissue (total area in which cells were counted was $0.032mm^2$).

3.3 Results

3.3.1 General anatomy of the Soay sheep pituitary gland

The average pituitary gland weight of the adult Soay rams (average body weight 36.0±1.5kg) was 595±39mg (mean±SEM, n=14). Of the pituitary gland caudal to the pars tuberalis, the pars distalis/zona tuberalis, pars intermedia and pars nervosa comprised on average 90%, 6.5% and 3.5% respectively of the entire pituitary gland.

The distribution of the various components of the pituitary gland is illustrated in Figures 3.1 and 3.2 in which sagittal and coronal sections of the rostral pituitary gland were stained for αMSH, ACTH, LHβ and prolactin. The general organisation of the pars intermedia is represented schematically in Figure 3.3. The pars intermedia extended into the midline of the dorsal face of the pars distalis and stained heavily for both αMSH and ACTH. ACTH expression was localised in

scattered cells of the pars distalis which were presumed to be corticotrophs. The pars intermedia and the pars distalis were largely separated by the residual lumen of Rathke's pouch, but frequent direct contacts between these two tissues were also observed. The pars intermedia adhered closely to the pars nervosa. At the rostral end, the pars intermedia was detached from the median eminence. LHβ-expressing gonadotrophs were scattered throughout the pars distalis, zona tuberalis and the pars tuberalis. By contrast, prolactin staining was restricted to the pars distalis. Within the pars distalis, lactotrophs were the most abundant cell type comprising 50%-60% compared with 7%-8% corticotrophs and 5%-8% gonadotrophs.

3.3.2 Cellular organisation of the pars intermedia and pars distalis

The appearance of the pars intermedia at high magnification is illustrated in Figure 3.4 and 3.5. Figure 3.4 demonstrates the close association between the pars intermedia and pars nervosa as well as the dissociation of the pars intermedia from the pars distalis. Figure 3.5 shows an area of direct contact between these tissues. The pars intermedia had a lobular organisation and contained finger like projections from the pars nervosa. The lining of the residual lumen on the side of the pars distalis contained a remarkable concentration of gonadotrophs (Figure 3.4b). Occasional gonadotrophs were also present in the pars intermedia while in the pars distalis the gonadotrophs had a particularly scattered distribution compared with the lobular association of the lactotrophs. The zona tuberalis was richly vascularised whereas by comparison scattered small blood vessels were observed in the pars intermedia.

3.3.2 Localisation of the site of expression of PC1 and PC2.

Immunocytochemical staining for PC1 and PC2 in the pars intermedia is illustrated in Figure 3.6. Low level of expression of PC1 was detected in a high population of the cells within the pars intermedia. By comparison, PC2 was more heavily expressed by the majority of the cells within the pars intermedia. For both enzymes, the staining was localised within the cytoplasmic compartment. Moreover, both αMSH and ACTH were detected in this tissue.

The pattern of expression of PC1 and PC2 in the pars distalis is illustrated in Figure 3.7. Immunostaining for PC1 and PC2 was detectable in a small and scattered population of cells within the pars distalis. PC2 staining in the pars distalis was weaker than that observed in the pars intermedia. ACTH was heavily expressed in a proportion of the pars distalis cells whereas only weak expression of αMSH was detected in this tissue.

3.4 Discussion

This immunohistological study has allowed the identification of several distinct tissues and mapped the distribution of many endocrine cell types by their secretory products in the Soay ram pituitary gland. This study identified five specific compartments within the pituitary gland of the Soay ram based on the distribution of endocrine cell types. These compartments were the pars tuberalis, the zona tuberalis, the pars distalis, the pars intermedia and the pars nervosa. These compartments are as described in other sheep breeds (Perry *et al.*, 1981; Polkowska *et al.*, 1980; Skinner and Robinson, 1995) and are conserved in many other mammalian species (Atwell, 1918; Harris, 1947; Wingstrand, 1966). As with other sheep breeds, the pars intermedia persists as a major pituitary gland tissue in the adult (Perry *et al.*, 1981).

Within the Soay pituitary gland, POMC-derived peptides were expressed in two pituitary tissues. These were the pars intermedia in which αMSH-expressing melanotrophs were the predominant cell type and the pars distalis which contained scattered, principally ACTH-expressing corticotrophs. The pars intermedia was also strongly immunoreactive for the ACTH antibody which cross-reacts (100%) with CLIP. Both these peptides are likely to contribute to this immunoreactivity in the pars intermedia since ACTH is an intermediate in αMSH and CLIP synthesis from POMC. The observation that ACTH is processed to yield αMSH in the pars intermedia of the Soay pituitary gland is consistent with immunocytochemical studies of the pars intermedia in the mouse (Marcinkiewicz *et al.*, 1993), the rat (Mains and Eipper, 1990) and the frog (Maruthainar *et al.*, 1992).

The pars intermedia expresses both PC1 and PC2, both of which are necessary for the biosynthesis of αMSH (Castro and Morrison, 1997; Mains and Eipper, 1990; Smith and Funder, 1988). The expression of both PC1 and PC2 in the pars intermedia implies that POMC is more extensively processed in this tissue than in the corticotrophs of the pars distalis. The expression of both PC1 and PC2 has been observed in the pars intermedia of the mouse (Marcinkiewicz *et al.*, 1993), the rat (Mains and Eipper, 1990) and the frog (Kurabuchi and Tanaka, 1997).

The principle POMC-derived product expressed by the corticotrophs of the pars distalis was ACTH. αMSH immunoreactivity in the pars distalis was weak compared to the pars intermedia and may be accounted for by the low crossreactivity of the αMSH antiserum with ACTH (0.15%). The crossreactivity of the ACTH antiserum for CLIP is not a relevant consideration in the pars distalis given the weak immunoreactivity for αMSH observed in this tissue. As αMSH and CLIP are both generated where ACTH is endoproteolytically cleaved, where a

tissue lacks αMSH immunoreactivity it will also not express CLIP. Thus, the corticotroph is unlikely to secrete significant amounts of αMSH in the Soay sheep. Similarly, αMSH expression in the adult pars distalis is low in the mouse (Marcinkiewicz *et al.*, 1993), the rat (Mains and Eipper, 1990) and the frog (Kurabuchi and Tanaka, 1997).

The pattern of PC1 and PC2 expression in the pars distalis adds further strength to the concept that the corticotroph does not secrete significant quantities of αMSH. PC1 is expressed in a small population of cells in the pars distalis with a similar distribution to those expressing ACTH. A small population of cells of the pars distalis stained weakly for PC2 but the intensity of staining was low compared to the pars intermedia. These PC2-expressing cells of the pars distalis may not be corticotrophs since in other species there are reports that PC2 is expressed in the pars distalis by gonadotrophs (Marcinkiewicz *et al.*, 1993). The low level of both αMSH and PC2 in the corticotroph is indicative of less extensive POMC-processing compared to the melanotroph in which αMSH and PC2 are strongly expressed. The distribution of PC1 and PC2 within the Soay pituitary gland is consistent with that observed in the cow (Egger *et al.*, 1994; Kirchmair *et al.*, 1992) and the mouse in which the precursor, POMC, is differentially processed in these two pituitary compartments to produce a tissue-specific range of peptides (Benjannet *et al.*, 1991; Marcinkiewicz *et al.*, 1993). In the mouse, in corticotrophs POMC is cleaved into ACTH, βLPH and possibly some βEND through the proteolytic action of PC1. Scattered corticotrophs are found throughout the pars distalis of the murine pituitary gland. However, PC2 and αMSH are co-expressed at a low level by a proportion of these cells in the mouse. The expression of PC2 and αMSH in the murine pars distalis is maximal in the immature animal, but persists in the adult (Marcinkiewicz *et al.*, 1993).

This study also identified an αMSH-secreting cell type in the pars tuberalis not associated with the pars intermedia. Although occasional corticotrophs have previously been observed in the pars tuberalis of the human pituitary (Baker, 1977), the cells observed in this study, unlike corticotrophs appear to produce significant amounts of αMSH since they are much more readily immunoreactive with the αMSH antiserum than are the corticotrophs of the pars distalis. These cells are few in number compared to the melanotrophs of the pars intermedia and are unlikely to contribute appreciably to peripheral levels of αMSH.

Overall, this study confirms the presence of two classic POMC-expressing cell types in the pituitary gland of the Soay ram. The pattern of expression of αMSH, a product of both PC1 and PC2-mediated POMC cleavage and ACTH, a product of PC1-mediated POMC cleavage alone, are consistent with the concept that the melanotroph is the major source of circulating αMSH. The pituitary gland

in the sheep expresses POMC at a level far exceeding that of any other tissue (Smith and Funder, 1988) and this study demonstrates unequivocally that the pars intermedia is the principle source of αMSH in the pituitary gland of the Soay sheep. Therefore it is evident that the conspicuous seasonal cycle of circulating αMSH previously observed in the Soay sheep is generated through the regulation of the activity of the pars intermedia. The seasonal regulation of the activity of the pars intermedia is the focus of the next study.

Figure 3.1. Immunocytochemical characterisation of the rostral region of the pituitary gland of the Soay ram in sagittal section using antisera for αMSH, ACTH, LHβ (LH) and prolactin (PRL). PI- pars intermedia, PD- pars distalis, ME- median eminence, PT- pars tuberalis. The pars intermedia was highly immunoreactive for αMSH (top left). Immunocytochemistry for ACTH (bottom left) revealed scattered immunoreactive cells in the pars distalis, presumably corticotrophs. The pars intermedia also stained positively for ACTH. LHβ-immunoreactive gonadotrophs were widely distributed and occurred singly throughout the pars distalis and occassionally in the pars tuberalis and the margins of the pars intermedia. Prolactin-immunoreactive lactotrophs (bottom right) were restricted to the pars distalis where their distribution was strikingly lobular. In the pars distalis lactotrophs occurred with a frequency greater than that of corticotrophs or gonadotrophs.

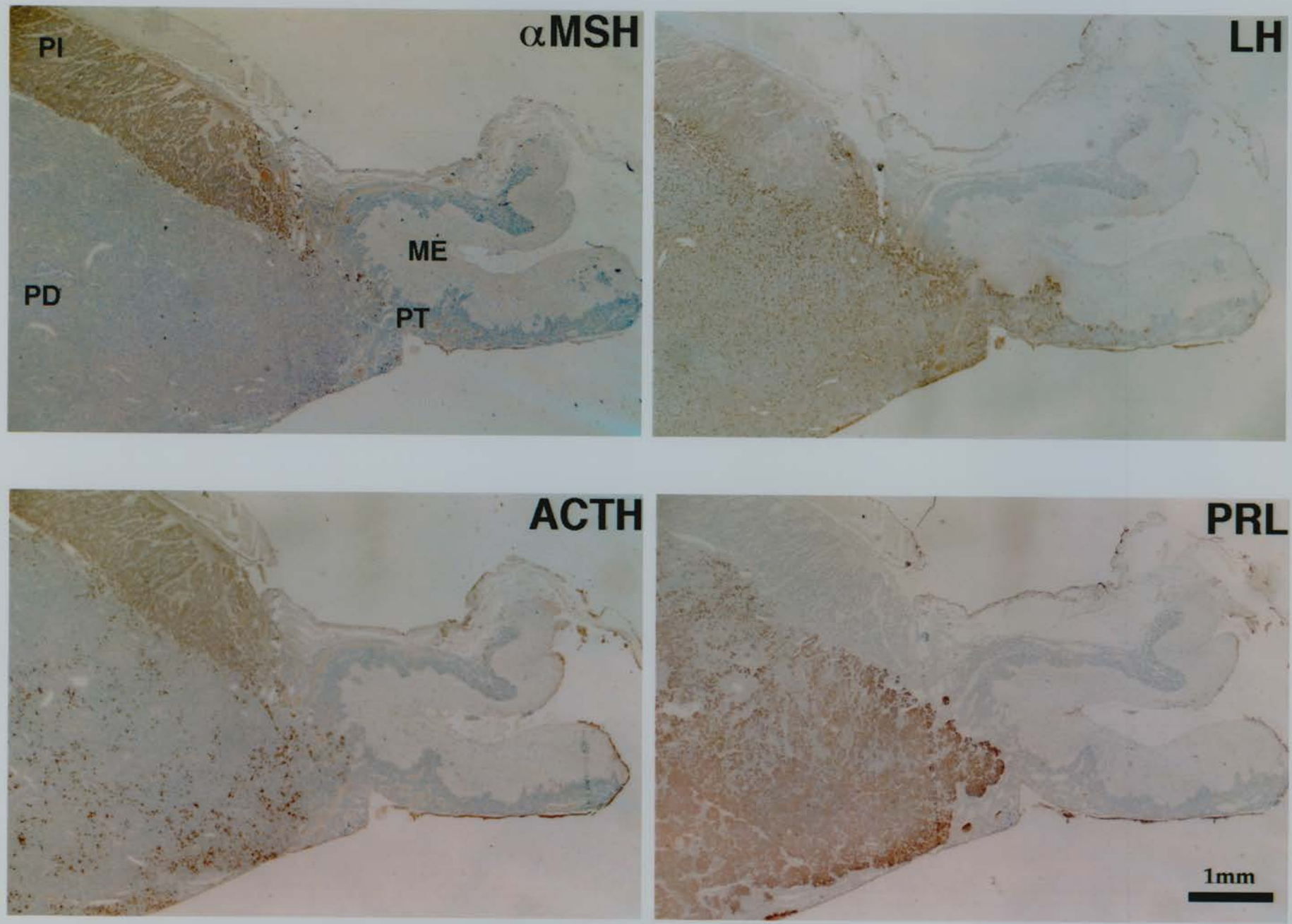
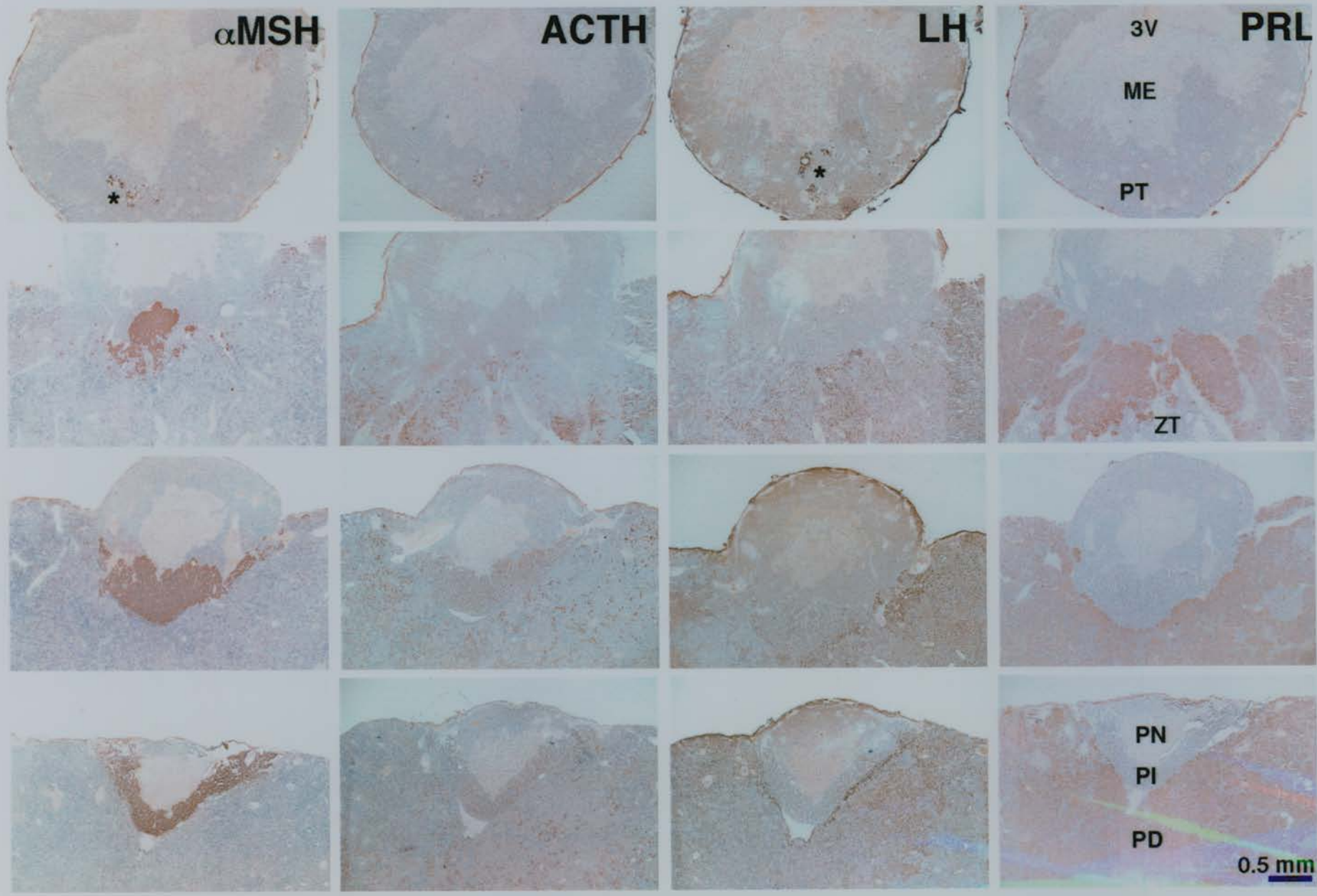

αMSH
PI
ME
PD
PT
LH
ACTH
PRL
1mm

Figure 3.2. Immunocytochemical characterisation of the rostral pituitary gland of the Soay ram through representative coronal sections. These sections were cut sequentially at 2mm intervals caudally to the pars tuberalis (top) to the medial pituitary gland (bottom). LH- LHβ, PRL- prolactin, ME- Median eminence, 3V- extension of third ventricle, PT- pars tuberalis, ZT- zona tuberalis, PN-pars nervosa, PI- pars intermedia, PD- pars distalis. Most rostrally, the pars tuberalis surrounded the median eminence. The pars tuberalis was rich in large sinusoidal blood vessels (arrows). Note the presence of cells immunoreactive for LHβ and αMSH (*) associated with blood vessels in the pars tuberalis. The pars intermedia expanded caudally from the pituitary stalk from a loose projection of αMSH-staining cells (second row) into a V-shaped stucture surrounding the pars nervosa (bottom row). The zona tuberalis which formed a caudal extension to the pars tuberalis (second row) stained for LHβ, but not prolactin (right) and contained a few ACTH immunoreactive cells. The pars distalis, which stained heavily for prolactin, increased in size caudally and was the predominant tissue of the medial pituitary gland.


αMSH
ACTH
LH
PRL
3V
ME
PT
ZT
PN
PI
PD
0.5 mm

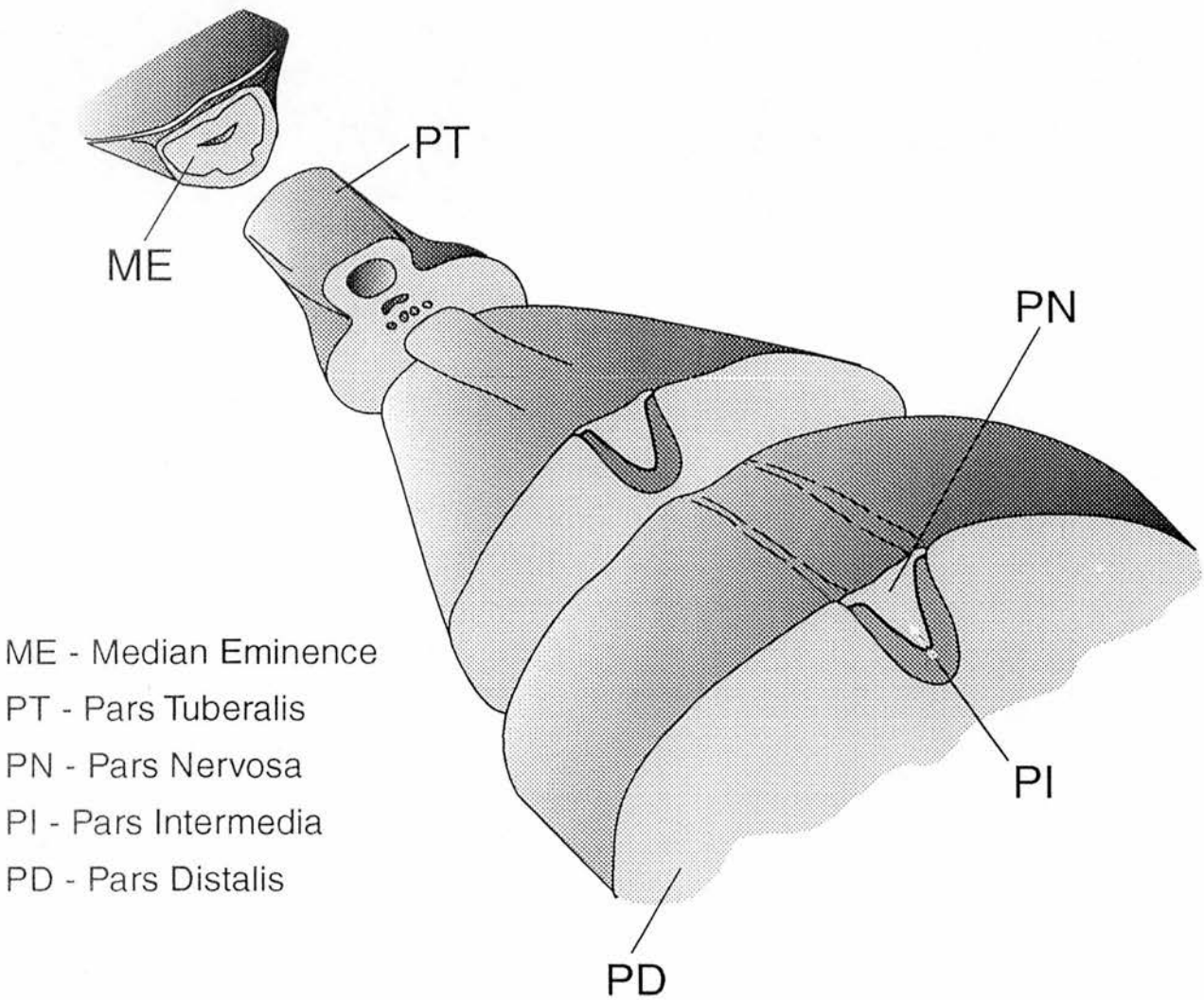
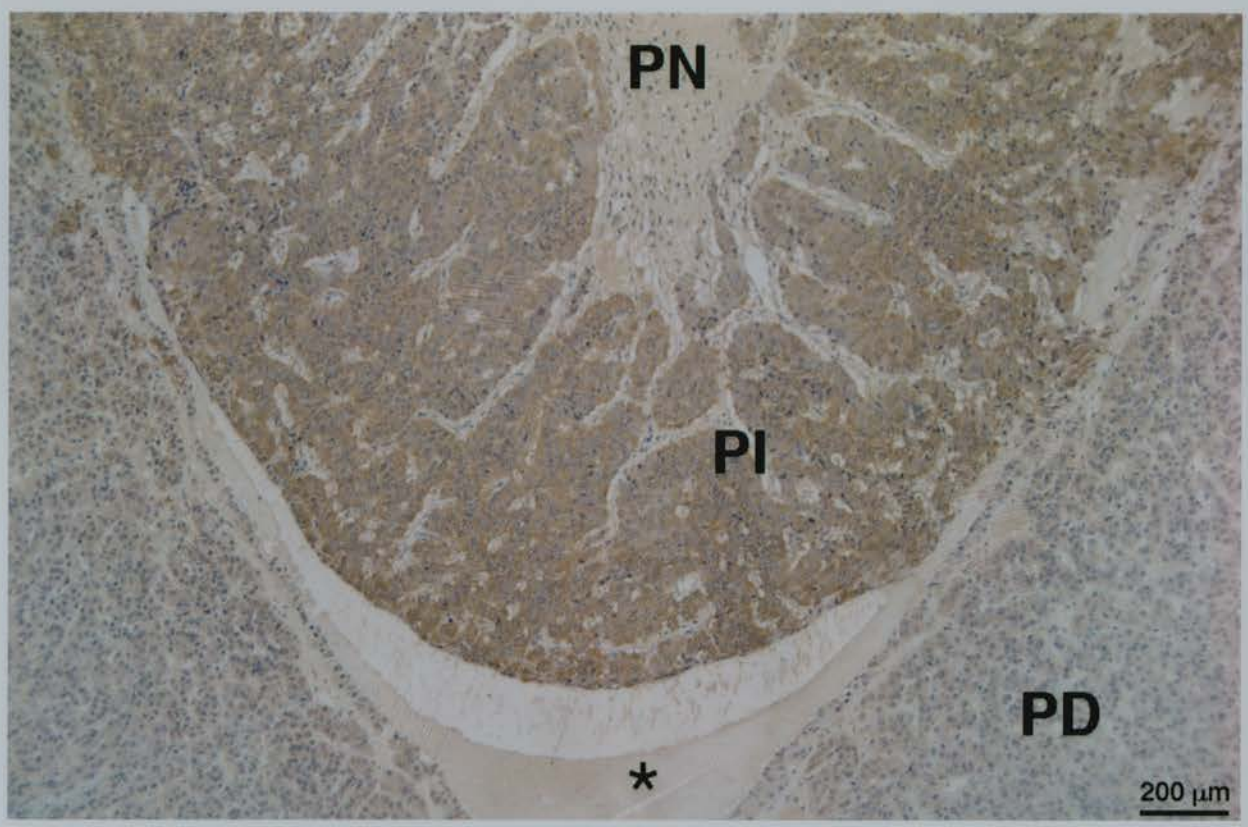


Figure 3.3. The organisation of the Soay ram pituitary from the pars tuberalis into the medial pituitary gland. This diagram is based on serial coronal sections of pituitary glands from a number of Soay rams. The pars tuberalis enclosed the median eminence which was continuous with the pars nervosa. The pars nervosa extended back into the pituitary gland, first lying very superficially on top of the dorsal surface before becoming progressively more embedded into the body of the pituitary gland. The pars intermedia separated the pars nervosa from the pars distalis except at its most rostral extent.

Figure 3.4. Coronal sections through the pars nervosa (PN), pars intermedia (PI) and pars distalis (PD) of the Soay ram.
Top panel; The pars intermedia immunocytochemically-stained for αMSH. The pars intermedia formed a V-shaped collar around the ventral and lateral margins of the pars nervosa along most of its length A clear residual lumen (*) is observed between the pars intermedia and the pars distalis.
Bottom panel; The margin between the pars intermedia and pars distalis, double immunocytochemically-stained for LHβ (HRP-DAB method, brown) and αMSH (APAAP-fast blue, blue). Immunocytochemical staining for αMSH in the pars intermedia, but not in the pars distalis, was clearly observed. Gonadotrophs were also observed in (arrows) and adjacent to the pars intermedia in a number of individuals.


PN
PI
PD
*
200 μm

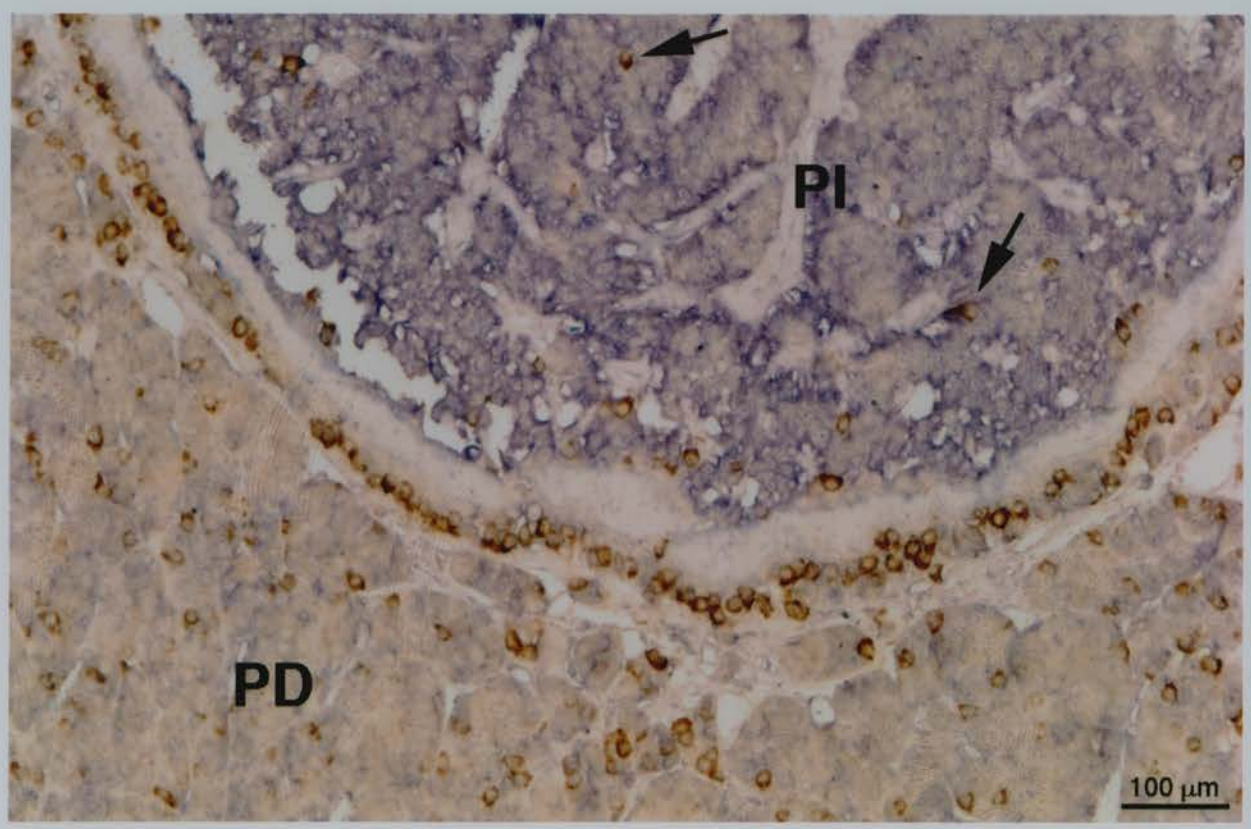

PI
PD
100 μm

Figure 3.5. The pars distalis (PD), pars intermedia (PI) and zona tuberalis (ZT) of the Soay ram at greater magnification.
Top panel; The pars intermedia and pars distalis immunocytochemically stained for prolactin (restricted to pars distalis) showing the boundary between these two tissues. The pars intermedia was clearly distinct from the pars distalis and was frequently separated from the pars distalis by the residual lumen of Rathke's pouch. Bottom panel; In contrast, there was no clear boundary between the zona tuberalis and the pars distalis. The zona tuberalis contained numerous sinusoidal blood vessels which frequently contained red blood cells.

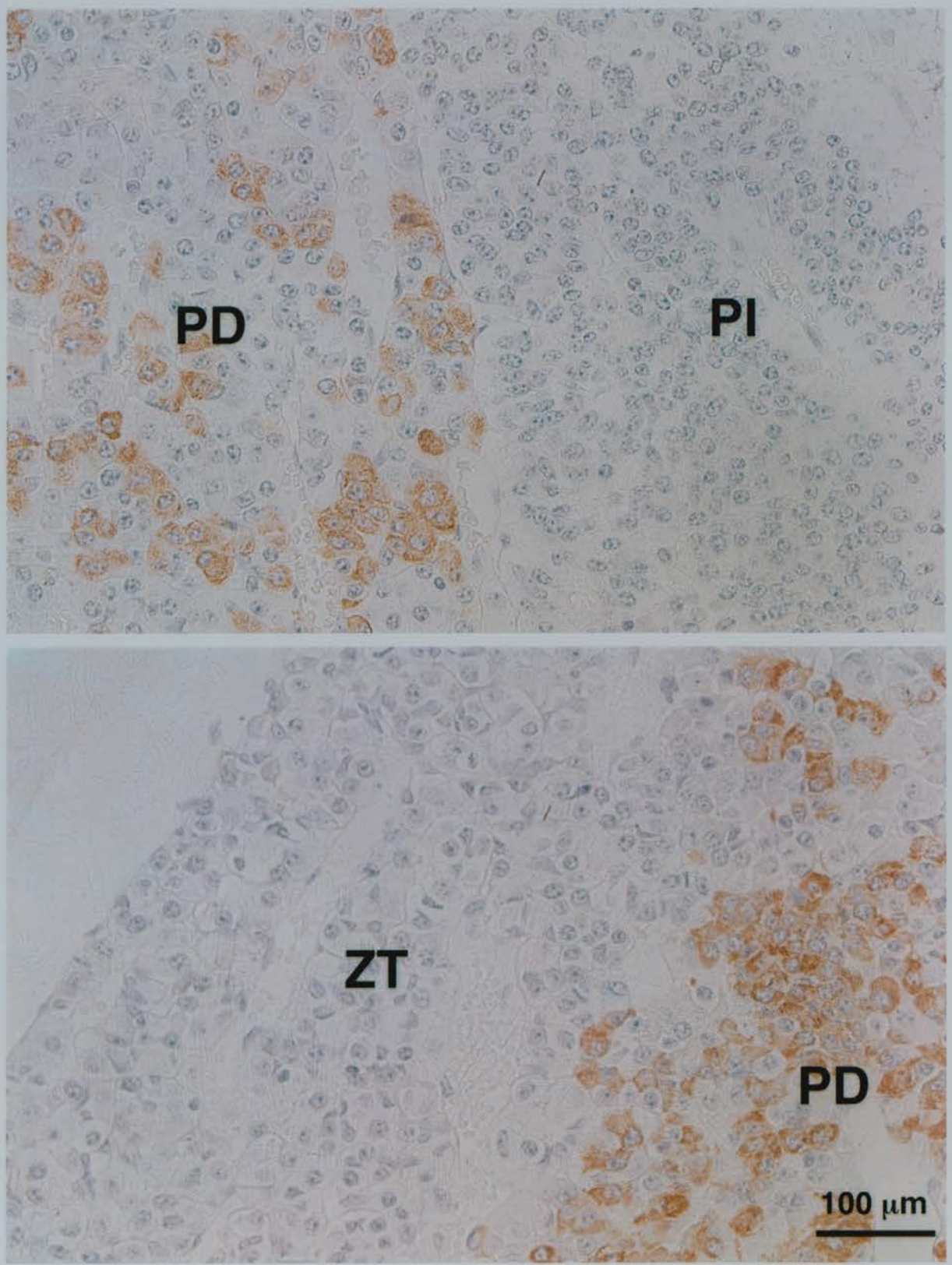

PD
PI
ZT
PD
100 μm

Figure 3.6. The expression of POMC-derived peptides and their endoproteolytic processing enzymes by the Soay ram melanotroph. Melanotrophs were strongly immunoreactive for both αMSH and ACTH (top). Immunocytochemistry for the processing enzymes PC1 and PC2 demonstrated that both enzymes are expressed by the melanotroph (bottom), although the staining for PC1 was weaker.

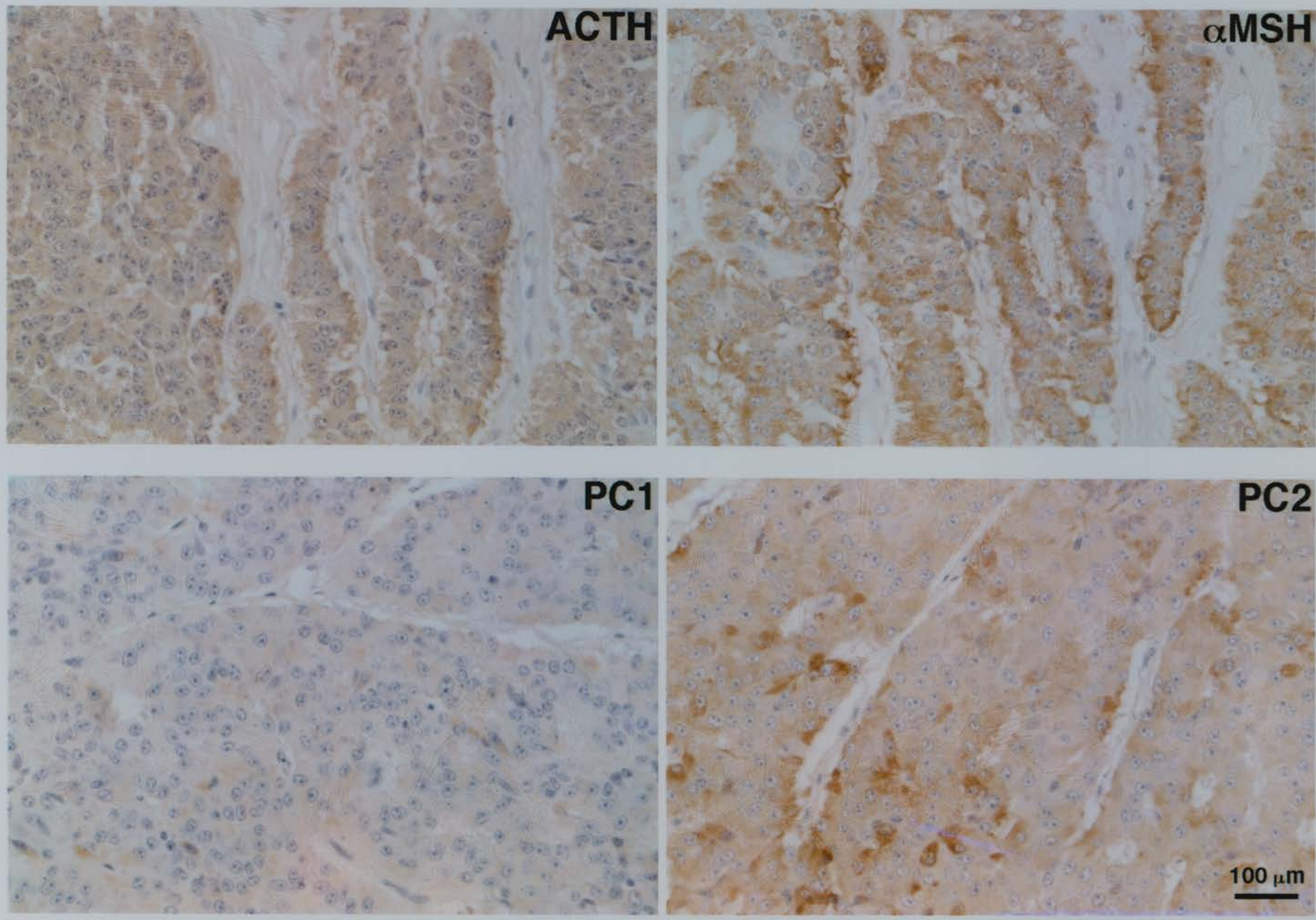
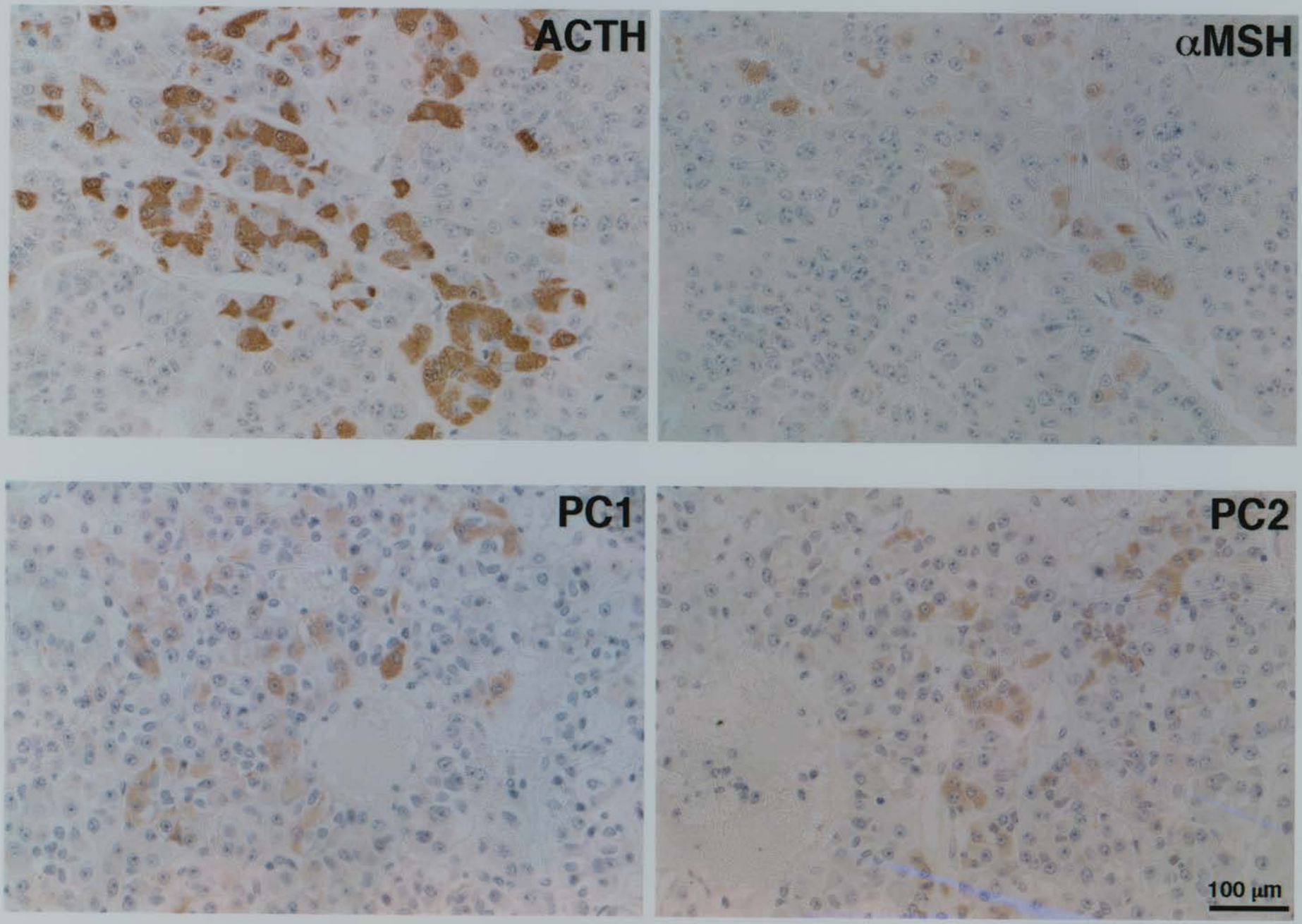

ACTH
αMSH
PC1
PC2
100 μm

Figure 3.7. The expression of POMC-derived peptides and their endoproteolytic processing enzymes by the Soay ram corticotroph. Corticotrophs expressed ACTH and stained weakly for αMSH (top). PC1 and PC2 (bottom) were also expressed by scatttered cells of the pars distalis, presumed to be corticotrophs, although the intensity of PC2 immunocytochemical staining was much lower than in the pars intermedia.


ACTH
αMSH
PC1
PC2
100 µm

Chapter 4

Seasonal regulation of pars intermedia POMC expression and processing.

4.1 Introduction

In the Soay sheep, circulating αMSH and βEND show a pronounced seasonal cycle in which αMSH is 3-20 fold higher in late summer and autumn than in winter and spring (Ebling and Lincoln, 1987; Lincoln, 1991). This cycle occurs in males, females and castrated males, and can be readily entrained by exposing animals to an artificial lighting regimen indoors (Lincoln and Baker, 1995). While the general endocrinology of this cycle has been described, the molecular and cellular events within the pars intermedia which generate the seasonal cycle in αMSH secretion in the sheep are unknown. Thus the aim of this study was to measure the differential expression of the mRNAs for POMC, PC1 and PC2 in the pars intermedia at different times of the year. It was anticipated that seasonally coordinated changes in the expression of all three genes would account for the observed changes in circulating concentrations of αMSH. This is supported by observations in the rat, in which pharmacological activation and inhibition of the pars intermedia induced changes in expression of POMC and the associated endoproteolytic processing enzymes (Oyarce *et al.*, 1996). To achieve these aims, we utilised the partial ovine cDNA sequences for PC1 and PC2 to characterise the level of expression of these mRNAs in the pituitary gland and brain. This was further confirmed by immunocytochemistry for the corresponding peptide products of these mRNAs. Moreover, the possibility of proliferative and cellular changes in autumn which result in hypertrophy of the pars intermedia and consequently the enhanced αMSH secretion was investigated.

4.2 Experimental procedures

4.2.1 Animal treatments and collection of tissues

Animals used in the seasonal study were Soay ewes born the previous spring (eight to nine months before commencing the experiment) and were maintained outdoors in grass paddocks in Fife, Scotland (56°N). The animals received supplementary feeding of commercial sheep nuts and hay during the winter (December to April). The animals were run with a ram from November to April and became pregnant around the

beginning of the study (Table 4.1). Groups (n=5) were culled at four times of year; approximately at the winter solstice (17th December, WS), the spring equinox (20th March, SE), the summer solstice (20th June, SS), and the autumn equinox (24th September, AE). The animals were transported to the Marshall Building near Edinburgh where they were allowed to acclimatise for three days before being killed. Immediately after death, the pituitary glands were separated, weighed and dissected into rostral and caudal portions (section 2.3.1). Tissues from the rostral part were promptly fixed for five hours in Bouins reagent and processed for histology as described in section 2.4. The remaining tissue from the caudal part was dissected into pars intermedia/pars nervosa and pars distalis and snap frozen and stored at -70°C until RNA extraction (section 2.3). These tissues were utilised in RNase protection assays, in situ hybridisation and immunocytochemistry.

Additional material from adult Scottish Blackface ewes held at the Moredun Research Institute was obtained at different times of the year. The pituitary gland, medio-basal hypothalamus (from the optic chiasm back to the mammilary bodies), latero-ventral hippocampus and cerebellum were removed and snap frozen and stored at -70°C until RNA extraction (section 2.3). These RNA were utilised in Northern blot analyses, although the effect of season was not studied in these animals.

Table 4.1 Data on whole body, pituitary gland and ovary weight as well as reproductive status in Soay ewes used in studies into the seasonal regulation of circulating POMC-derived peptides.

Animal	Body Wt (Kg)	Pituitary Wt (mg)	Ovary Wts (L/R; mg)	Comments
WS1	16.8	-	589/518	Pregnant-early
WS2	15.0	226	233/407	Pregnant-early
WS3	16.4	282	925/342	Pregnant-early
WS4	15.6	326	1183/353	Pregnant-early
WS5	15.8	273	730/215	Pregnant-early
SE6	15.2	312	785/343	Pregnant-late
SE7	19.4	281	297/827	Pregnant-late
SE8	16.4	351	278/974	Pregnant-late
SE9	16.3	295	178/788	Pregnant-late
SE10	18.2	335	178/827	Pregnant-late
SS11	19.3	436	372/384	Lactating-early
SS12	18.1	395	328/414	Lactating-early
SS13	24.0	468	574/560	Lactating-early
SS14	20.0	432	278/528	Lactating-early
SS15	24.7	441	503/573	Non-lactating
AE16	25.0	400	468/522	Lactating-late
AE17	23.2	301	532/718	Lactating-late
AE18	25.0	333	415/313	Lactating-late
AE19	21.0	341	476/627	Lactating-late
AE20	24.0	337	574/610	Lactating-late

Abbreviations: WS, winter solstice group; SE, spring equinox group; SS, summer solstice group; AE, autumn equinox group.

4.2.2 RNase protection assay

RNase protection assays were carried out for POMC, PC1 and PC2 mRNAs on 5μg total pars intermedia RNA (section 2.13) using cRNA probes generated from the subcloned ovine cDNA sequences described in detail in section 2.8. The size of the protected fragments was assessed by comparison with RNA size standards (Ambion). The relative optical density of POMC, PC1 and PC2 RNA and 18S ribosomal standard were determined on a phosphorimager (Molecular Dynamics) and the 18S ribosomal standard was used to correct for loading variations in the RNA between individuals. Differences between the relative density in the different treatment groups were evaluated using ANOVA. Where ANOVA indicated that standard deviations differed between treatments, the natural logarithms of data were used for parametric comparisons. Pairwise comparisons between individual groups were carried out using the unpaired student t-test.

4.2.3 In situ hybridisation

In situ hybridisation was carried out for POMC, PC1 and PC2 mRNAs (section 2.12). In situ hybridisation employed the same ovine cDNA sequences as RNase protection assay (section 2.8). The tissue sections were exposed to photographic emulsions for a period ranging from 1-2 days (POMC) to six weeks (PC1 and PC2; section 2.11.6). Semi-quantitative analyses were undertaken using computer-aided image analysis (section 2.11). Analysis of variance was applied to grain density measurements obtained by image analysis between treatments. As with RNase protection assay, transformed data was used where the standard deviation differed between treatments.

4.2.4 Northern blot analysis

Northern blot analyses were carried out as described in section 2.10 using the ovine cDNA sequences (section 2.8) and RNA collected from the Scottish Blackface ewes. This gave general information on mRNA transcript sizes and extra-pituitary sites of expression of POMC and the processing enzymes.

4.2.5 Immunocytochemistry

Immunocytochemical staining for αMSH, ACTH, PC1 and PC2 was carried out on 3μm coronal sections of each pituitary gland (Section 2.5.4). Where semi-quantitative analysis of immunocytochemical staining intensity was made, sections were examined without prior knowledge of treatment group and staining intensity was graded from zero (antigen not detectable) to five (heavy immunostaining).

To measure pars intermedia cellular proliferative activity, immunocytochemistry for the proliferation marker, PCNA, was undertaken (Section 2.5.4). Percentage positive nuclear-staining and cell density measurements were carried out simultaneously by counting total and positively-staining nuclei within ten graticule units (Graticules Ltd, Tonbridge, UK) in ten different areas of the tissue (total area in which cells were counted was $0.032mm^2$). Counting of cells was made on slides without prior knowledge of treatment group and comparisons between groups were made using ANOVA. The cross-sectional area of the pars intermedia, pars distalis and pars nervosa was measured using an image analysis program (Proplus software).

4.3 Results

4.3.1 Animals

Average whole body weight in the Soay ewes increased significantly ($p<0.0001$) from winter (15.9±0.3kg; mean±SEM) to the following autumn (AE, 23.6±0.7kg). Pituitary gland weight also increased from winter (WS, 277±21; SE, 315±13mg) to summer (SS, 434±12 mg; $p<0.0001$) but then declined from summer (SS, 434±12 mg) to autumn (AE, 342±16mg; $p<0.01$).

4.3.2 POMC expression

POMC RNA transcripts of 1.2 and 2.8kb were detected in the pituitary gland. The 1.2kb transcript was the markedly predominant transcript. By comparison, only the 1.2kb transcript was detected in the mediobasal hypothalamus. No expression of POMC was detected by Northern blot hybridisation in the hippocampus or the cerebellum (Figure 4.1).

RNase protection assay (Figures 4.2 and 4.3) demonstrated significant seasonal variation ($P<0.01$) in POMC mRNA expression in the pars intermedia. POMC mRNA expression (Figure 4.3a) was lowest in winter (WS; 1.081±0.215) and spring (SE; 0.249±0.070) and increased in summer (SS; 1.252±0.547) and autumn (AE- 3.169±0.885; Optical density POMC protected fragment/18S protected fragment, arbitary units). Similarly, semi-quantitative analysis by in situ hybridisation (Figure 4.4) demonstrated significant seasonal variation in POMC mRNA expression in the pars intermedia ($P<0.01$). POMC expression was lowest in the winter (WS; 0.032±0.001) and spring (SE; 0.035±0.003) and increased in the summer (SS; 0.045±0.005) and autumn (AE- 0.050±0.003; Silver grain density, arbitary units; Figures 4.4 and 4.5a). POMC mRNA was also detected in the pars distalis (Figure 4.6). However, no effect of season detected on the level of expression in the pars

distalis (WS, 0.010±0.001; SE, 0.011±0.003; SS, 0.008±0.003; AE, 0.008±0.002; Figures 4.5b and 4.6).

4.3.3 PC1 expression

PC1 mRNA transcripts of 2.5kb were detected in the pituitary gland, the mediobasal hypothalamus, the hippocampus and the cerebellum. Additionally, minor transcripts of 1.8, 1.4 and 0.8kb were detected in the pituitary gland (Figure 4.7).

RNase protection assay confirmed expression of PC1 in the ovine pars intermedia (Figure 4.2). PC1 mRNA expression was increased in the pars intermedia in autumn compared to the rest of the year (Figure 4.3b; WS, 0.036±0.002; SE, 0.028±0.003; SS, 0.033±0.003; AE, 0.066±0.007; Optical density PC1 protected fragment/18S protected fragment, arbitary units; $p<0.001$).

PC1 mRNA was detectable by in situ hybridisation in both the pars intermedia (Figure 4.4) and the pars distalis (Figure 4.6). PC1 mRNA was undetectable in the pars nervosa (Figure 4.4). In the pars intermedia, there was a tendency for increased PC1 mRNA expression in autumn although this did not reach statistical significance (WS, 0.0176±0.0059; SE, 0.0357±0.0260; SS, 0.0162±0.0130; AE, 0.0781±0.0308; Silver grain density, arbitary units; Figure 4.9a). The temporal pattern of PC1 expression indicated by in situ hybridisation was similar to that observed by RNase protection assay.

PC1 peptide was detectable by immunocytochemistry in the pars intermedia of all animals (Figure 4.8). However, PC1 immunoreactivity was present in lower concentrations in the pars intermedia than in the adjacent pars nervosa. Again, there was a tendency for increased PC1-immunostaining in the pars intermedia from winter to the following autumn; however this did not reach statistical significance (WS, 0.4±0.2; SE, 0.6±0.2; SS, 0.8±0.2; AE, 1.5±0.5; Relative staining intensity, arbitrary units; Figure 4.8). Individual cells of the pars distalis also showed PC1 immunoreactivity and similarly this showed an effect of season just short of significance, with maximum staining intensity in animals killed at the spring equinox (WS, 1.4±0.2; SE, 2.8±0.4; SS, 2.2±0.4; AE, 2.0±0.3; P=0.06). PC1 staining in the neural lobe was unaffected by season (WS, 3.0±0.6; SE, 2.0±0.3; SS, 2.6±0.5; AE, 1.8±0.4; Figure 4.8).

4.3.4 PC2 expression

PC2 mRNA transcripts of 2.7kb were detected in the pituitary gland, the mediobasal hypothalamus and the hippocampus (Figure 4.10).

RNase protection assay confirmed the expression of PC2 mRNA in the pars intermedia (Figure 4.2). There was no effect of season on pars intermedia PC2 mRNA

expression (Figure 4.3; WS, 6.31±1.27; SE, 4.36±0.91; SS, 10.66±3.62; AE, 7.30±1.81; Optical density PC2 protected fragment/18S protected fragment, arbitary units). PC2 expression was detectable also in the pars intermedia by in situ hybridisation (Figure 4.4). There was a tendency for increased PC2 mRNA expression in autumn; however, due to the large individual variation this did not reach statistical significance (Figure 4.9b; WS, 0.0120±0.0058; SE, 0.0045±0.0035; SS, 0.0127±0.0110; AE, 0.0499±0.0385; Silver grain density, arbitary units). PC2 mRNA was not detected in the pars distalis (Figure 4.6).

PC2 peptide was detected in the pars intermedia of all animals studied (Figure 4.8). Additionally, some staining in the pars nervosa (Figure 4.8) and weak staining in isolated cells of the pars distalis (as described in the previous chapter, Figure 3.7) was observed. PC2 immunocytochemical staining intensity was unaffected by season in any of the three pituitary compartments studied (Pars intermedia; WS, 3.6±0.2; SE, 2.8±0.4; SS, 2.6±0.4, AE; 2.5±0.5; Pars Distalis; WS, 0.4±0.2; SE, 0.6±0.2; SS, 0.2±0.2; AE, 0.2±0.2; Pars Nervosa; WS, 2.0±0.3; SE, 1.2±0.2; SS, 0.8±0.4; AE, 1.6±0.4; Relative staining intensity, arbitrary units).

4.3.5 Pars intermedia αMSH and ACTH expression

The POMC-derived peptide αMSH was detected in the pars intermedia by immunocytochemistry at all times of the year (Figure 4.8). The intensity of αMSH staining was not significantly affected by season (WS, 3.8±0.4; SE, 3.8±0.2; SS, 3.6±0.5; AE, 3.8±0.6; Relative staining intensity, arbitrary units). ACTH immunoreactivity was also present in the pars intermedia with no differences between seasons. In the pars distalis, both αMSH and ACTH were present, although ACTH staining was more intense than αMSH as described in the previous chapter (Figure 3.7). The intensity of αMSH staining in the pars distalis was not altered with season.

4.3.6 Pars intermedia cell proliferation and morphology

Immunocytochemistry for PCNA revealed a significant increase in cell proliferation in the pars intermedia from winter to the following autumn (Figure 4.11a; $p<0.05$). The percentage of cells staining positively for PCNA increased from approximately 0.15% to 0.50% from the winter solstice to the autumn equinox (WS, 0.137±0.091; SE, 0.139±0.075; SS, 0.388±0.157; AE, 0.556±0.089%).

Pars intermedia cell density showed a highly significant decline from winter to the following autumn concomitant with the increase in PCNA expression (Figure 4.11b; WS, 1708±48; SE, 1182±126; SS, 1127±90; AE, 945±74 cells in 0.032mm^2; $p<0.0001$) indicative of an increase in cytoplasmic volume. In addition, there was a parallel increase in pars intermedia cross-sectional area (WS, 428990±97291; SE,

446776±58342; SS, 796788±55552; AE, 751459±49918μm^2; $p<0.01$). A increase in the cross-sectional area of the pars distalis was detected from winter to summer (WS, 12109000±2031000; SE, 16072000±1923000; SS, 23578000±3348000; AE, 20343000±3348000μm^2; $p<0.05$). No difference in cross sectional area between seasons was detected in the pars nervosa (WS, 577596±173298; SE, 341766±29199; SS, 378840±34579; AE, 379563±31402)

4.4 Discussion

This study clearly demonstrates that there are seasonal changes in the expression of POMC mRNA with an increase from spring to autumn in Soay ewes. In addition, there is evidence for parallel changes in the mRNA expression of the processing enzymes PC1 and PC2 although these changes are not statistically significant for PC2 due to the large individual variations between animals. This general result is consistent with the view that the seasonal cycle in αMSH secretion which is elevated in the late summer and autumn (Lincoln, 1991; Lincoln and Baker, 1995) results from the increased expression of the POMC precursor gene and the genes encoding the key processing enzymes involved in endoproteolytic cleavage of the POMC precursor molecule. The observation that both PC1 and PC2 are expressed in the pars intermedia is indicative of the notion that POMC is extensively processed in this tissue to yield primarily αMSH (Castro and Morrison, 1997; Smith and Funder, 1988). In the rat, experimental activation of the pars intermedia is associated with an increase in the expression both of POMC and its processing enzymes, including PC1 and PC2 (Oyarce *et al.*, 1996). In addition, in the frog adaptation to dark background which promotes αMSH secretion from the pars intermedia, is associated with up-regulation of multiple genes involved in αMSH synthesis and processing (Ayoubi *et al.*, 1992; Braks *et al.*, 1992; Maruthainar *et al.*, 1992). Overall, this is consistent with the view that the seasonal activation of the pars intermedia which results in increased circulating αMSH at the onset of winter, involves the coordinated regulation of the POMC and processing enzyme genes.

The results reported in this chapter also indicate that the seasonal variation POMC expression and αMSH secretion are accompanied by a change in the cell size of the proliferative activity of the pars intermedia. In autumn, when POMC mRNA expression is maximal, and peak circulating αMSH concentrations are reported (Lincoln and Baker, 1995), the pars intermedia undergoes hypertrophy and hyperplasia. Moreover, the highest cross-sectional area of the pars intermedia, the highest index of cell proliferation and the lowest cell density were all associated with maximal POMC mRNA expression. In the rat, inhibition of αMSH secretion from the pars intermedia using dopaminergic drugs is associated with a parallel decrease in the

proliferative index of the pars intermedia (Chronwall *et al.*, 1987). Moreover, in the sheep, disconnection of the pituitary gland from the primarily inhibitory influence of the hypothalamus results in an increase in αMSH secretion and hypertrophy of the pars intermedia (Clarke *et al.*, 1986). The study described here indicates that the pars intermedia is a dynamic tissue in the intact sheep waxing and waning in activity with season. The seasonal activation of the pars intermedia is likely to be a reversible effect since dopaminergic drugs inhibit proliferative activity and gene expression of POMC and its related processing enzymes as occurs in the winter (Chronwall *et al.*, 1987; Oyarce *et al.*, 1996). This aspect is discussed in more detail in the following experimental chapters.

The animals used in this study were juvenile at the onset of the study and thus the experimental observations are confounded by the effect of age. The increase in pituitary gland weight and the increase in the size of the pars intermedia (as indicated by cross-sectional area) were associated with an increase in overall body weight. This may be related to age rather than season since the pars intermedia of the sheep is reported to increase from a thickness of 10 cells in the neonate to 15 cells deep in the adult (Perry *et al.*, 1982). The pars distalis also showed an increase in cross-sectional area from winter to summer, probably also due to somatic growth. However, the weights of the pituitary glands were decreased in the autumn compared with summer which indicates an effect of season rather than age. This may be attributed to a decline in the size of the pars distalis associated with regression of the activity of the lactotrophs (Lincoln and Baker, 1995; Lincoln and Clarke, 1994). The treatment groups in this study do not allow for more accurate dissection of the effect of season and age on the functional activity of the pars intermedia. The data presented in this study may also be affected by pregnancy and stress caused by transport three days before killing. Pregnancy and lactation are known to delay the seasonal maximum in circulating αMSH concentrations by approximately two months (Lincoln and Baker, 1995).

Northern blot analysis indicated that two POMC transcripts of 1.2kb and 2.8kb were expressed in the ovine pituitary gland. The 1.2kb transcript was the more dominant. Transcripts of 1.25 to 1.4 kb have been reported in the pituitary gland of the fetal sheep (Merei *et al.*, 1993). PC1 mRNA transcripts of several sizes were detected. Multiple transcription start sites are reported in the human PC1 gene (Jansen *et al.*, 1995). However, the smaller of these (less than 2.0kb), which were only detected in the pituitary gland, may be degradation products since they are likely to be too small to code for functional PC1 based on the cDNA sequences of other species (Bloomquist *et al.*, 1991).

This study describes the localisation of the mRNA for PC1 and PC2 in the sheep pituitary gland for the first time. The exposure times needed for detection of POMC and PC1/PC2 signals in the in situ hybridisation studies suggest that either the POMC

gene is comparatively a more heavily expressed gene and/or the POMC RNA is a more stable transcript than that of the processing enzymes. POMC and PC1 mRNA were observed both in the pars intermedia and the pars distalis whereas PC2 mRNA was not detectable in the pars distalis of any animals although immunocytochemistry indicated that PC2 is expressed at low levels. These observations further substantiate the findings in the previous chapter which indicated that PC2 is principally expressed by the pars intermedia. Since PC2 is thought to be necessary for the efficient synthesis of αMSH (Castro and Morrison, 1997; Smith and Funder, 1988), these observations further implicate the pars intermedia as the principal source of circulating αMSH.

PC1 and PC2 peptides were also detected in the pars nervosa of the Soay sheep. PC1 and PC2 have previously been reported in the bovine pars nervosa (Egger *et al.*, 1994). However, RNA transcripts for these enzymes were not detected in the pars nervosa suggesting that these enzymes are transported to the axons and nerve terminals of the pars nervosa from the cell bodies in the hypothalamus. These enzymes may be involved in the endoproteolytic processing of secretogranin II to yield secretoneurin. Secretogranin II mRNA is also restricted to the neuronal cell bodies in the hypothalamus (Egger *et al.*, 1994).

The novel ovine PC1 and PC2 sequences generated for this study both show considerable nucleotide and predicted peptide sequence homology to their murine and human counterparts (Bloomquist *et al.*, 1991; Dai *et al.*, 1995; Seidah *et al.*, 1992). PC1 and PC2 mRNA transcripts are expressed in tissues that do not express POMC, such as the pars nervosa and the hippocampus, indicating that these endoproteases act on a number of substrates. The diversity of substrates for the prohormone convertases is illustrated by the multiple phenotypical effects of mutations of these enzymes. Mutations in PC1 have been reported in the human (O'Rahilly, 1998) and resulted in multiple phenotypical effects which included early onset obesity (likely to be caused by the failure to process insulin and possibly POMC) and abnormal pigmentation (likely to be due to impaired POMC processing resulting in a lack of functional αMSH at the melanocyte). The high conservation of sequences and widespread expression for these enzymes reflects the fundamental role played by these enzymes in many physiological systems.

This study has clearly demonstrated coordinate changes in the expression of the mRNAs for POMC and at least one of its processing enzymes with season. However, it is not clear how much of this apparently seasonal regulation of the expression of these mRNAs is actually attributable to age and somatic growth. The effect of photoperiod (which is the most widely used physiological indicator of season) on pars intermedia function is the focus of the next study.

Figure 4.1 Northern blot analysis on 5μg total RNA probed with radiolabelled POMC cDNA. POMC mRNA transcripts of 1.2kb (major transcript) and 2.8kb (minor transcript) were detected in RNA extracted from the pituitary gland (Pt). The 1.2kb transcript is also expressed in RNA extracted from the mediobasal hypothalamus (MBH). POMC does not appear to be expressed in the hippocampus (Hp) or the cerebellum (Cb). Exposed to X-OMAT LS film for 4 days.

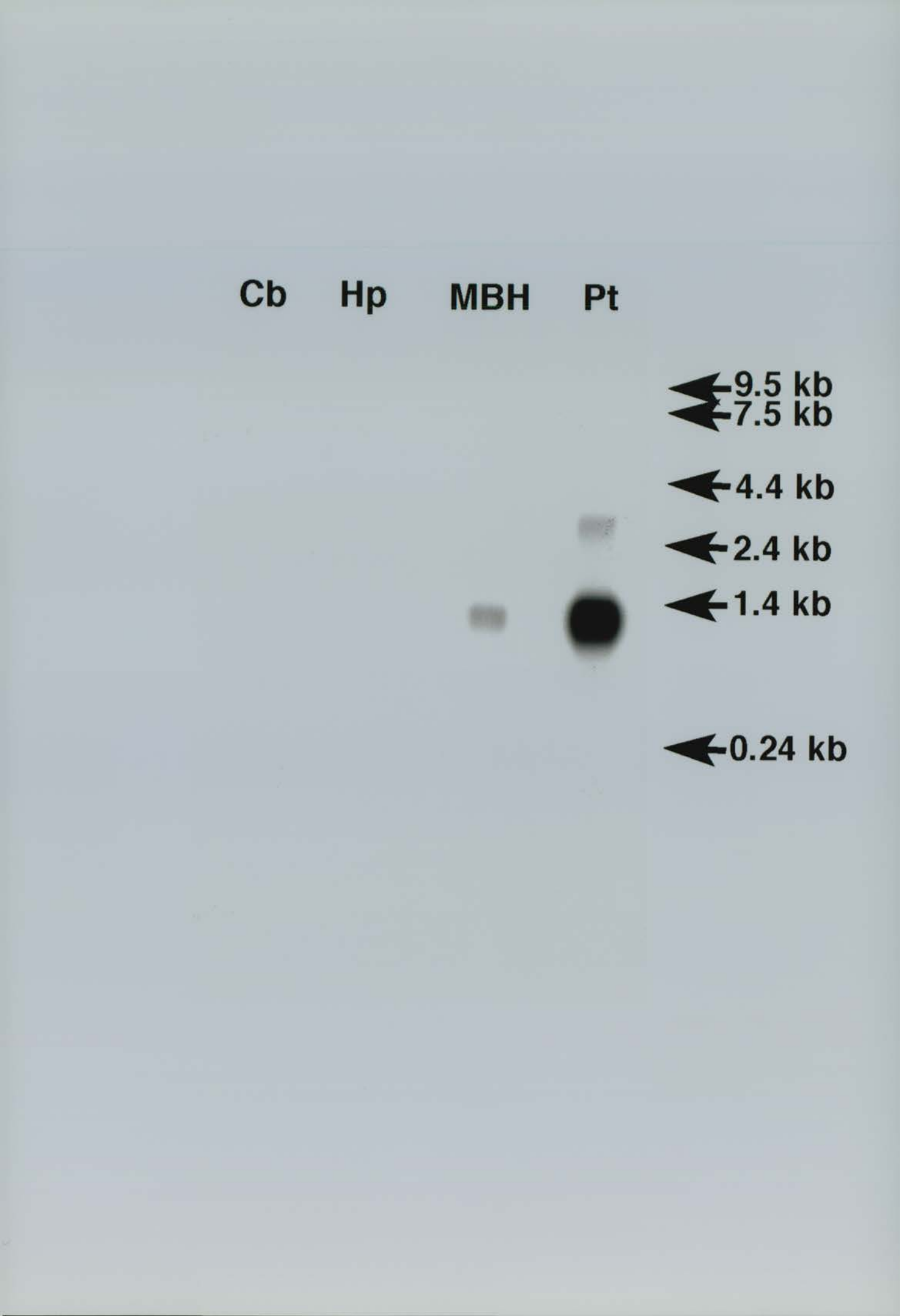
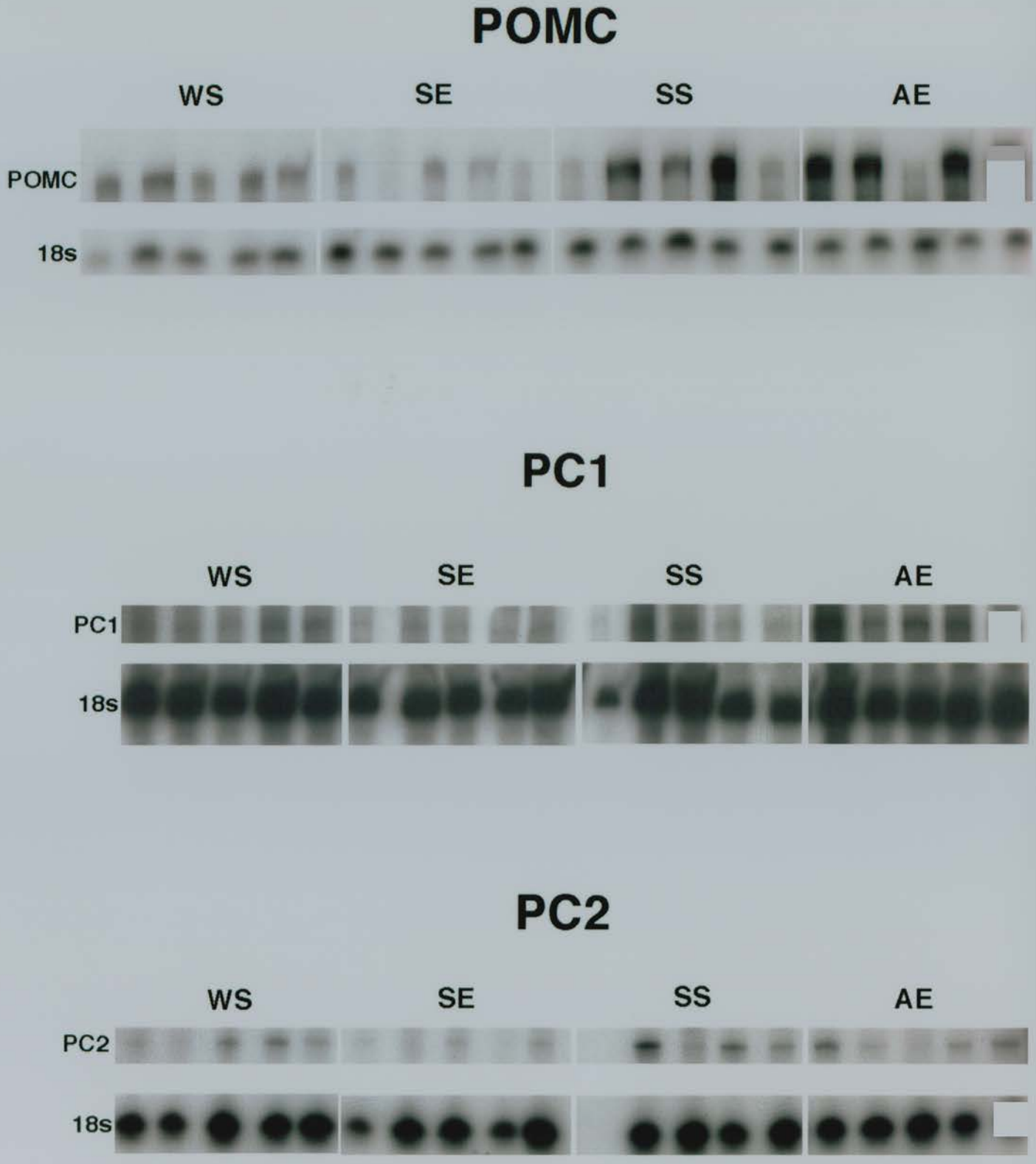

Cb
Hp
MBH
Pt
9.5 kb
7.5 kb
4.4 kb
2.4 kb
1.4 kb
0.24 kb

Figure 4.2 RNase protection assays for POMC, PC1 and PC2 mRNA extracted from the pars intermedia. Protected cRNA probe/mRNA band (top) and 18s (internal control) band (bottom). Exposed for 2 days (POMC) or 2 weeks (PC1 and PC2). WS- Winter solstice, SE- Spring equinox, SS- Summer solstice, AE- Autumn equinox.


POMC
WS
SE
SS
AE
POMC
18s
PC1
WS
SE
SS
AE
PC1
18s
PC2
WS
SE
SS
AE
PC2
18s

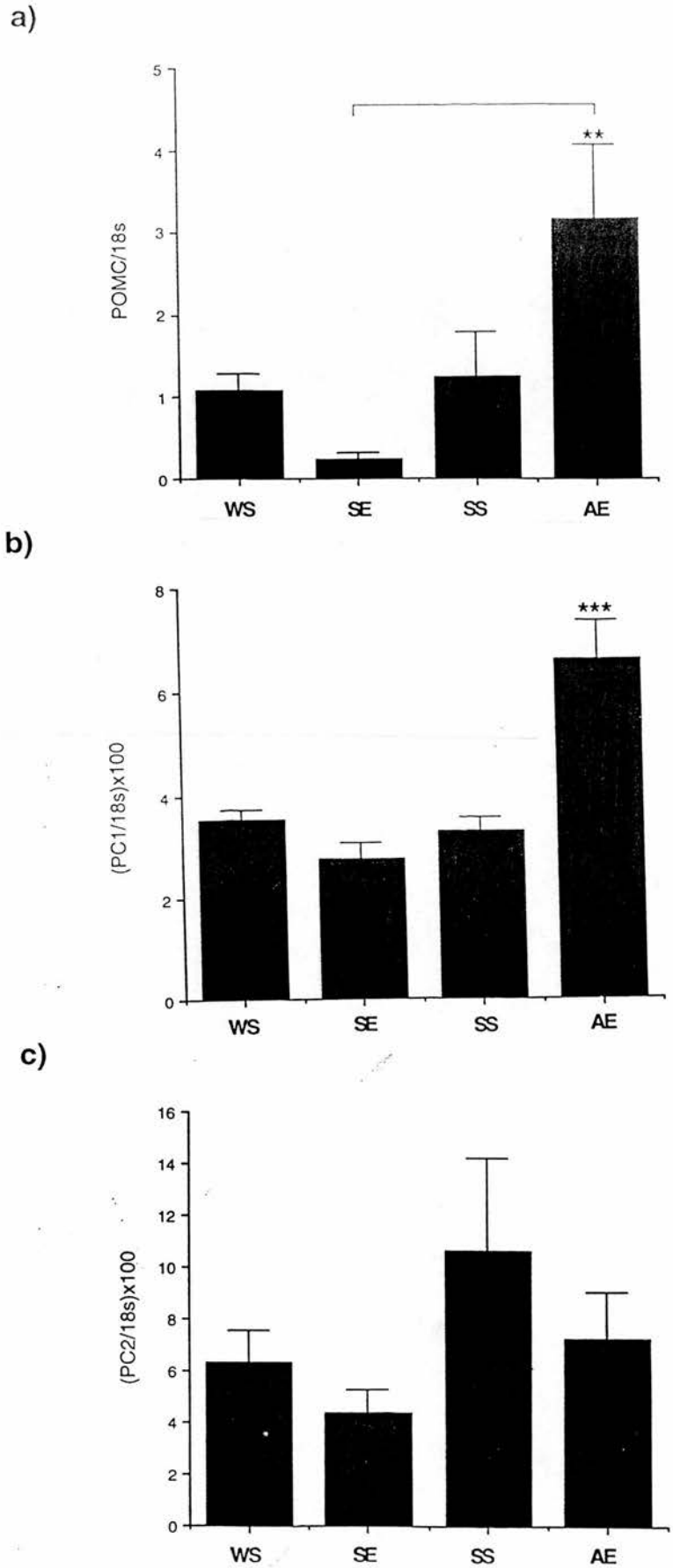
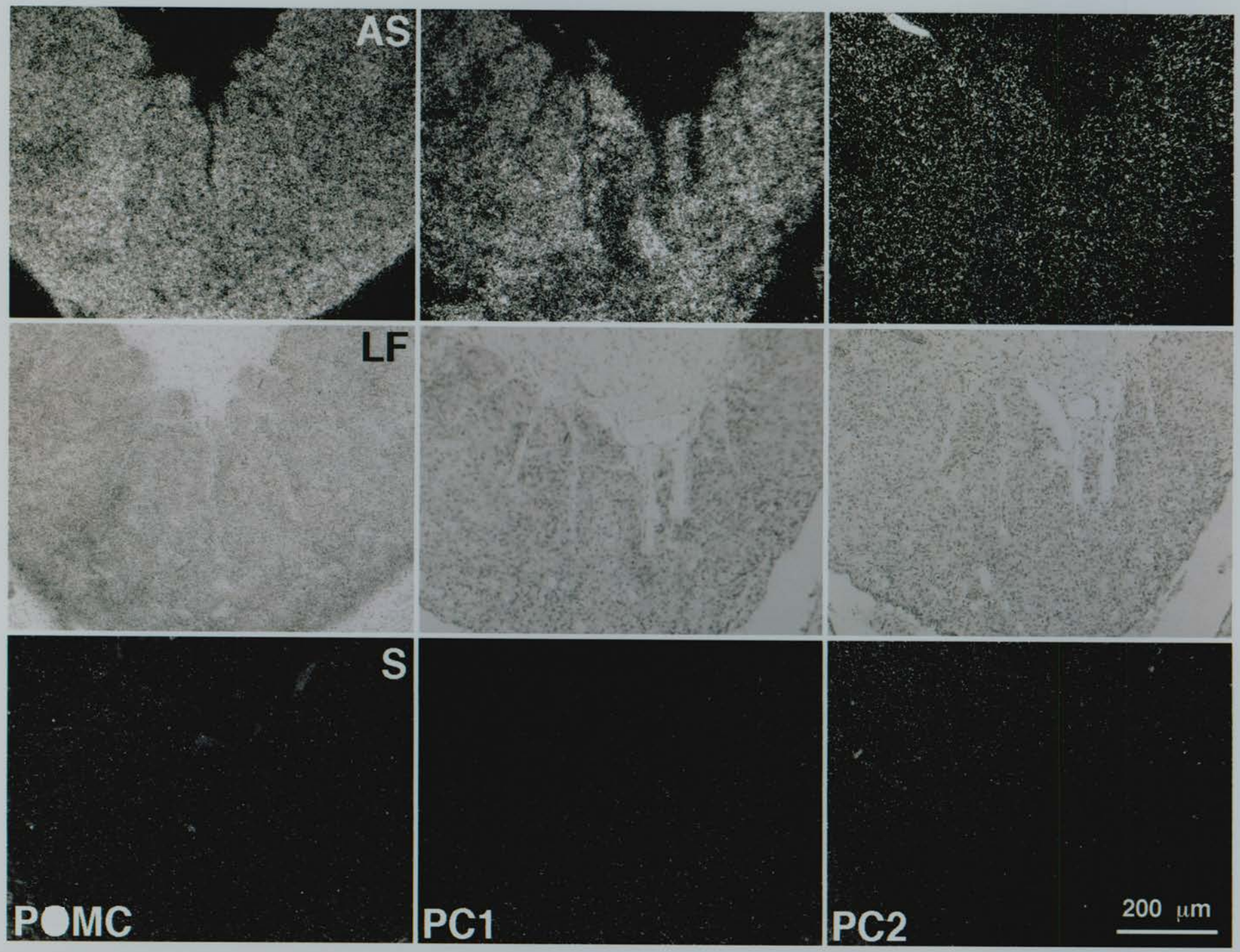


Figure 4.3 Quantitative analysis (intensity of protected fragment divided by intensity of 18s internal control) of POMC (a), PC1 (b) and PC2 (c) RNase protection assays on mRNA extracted from the pars intermedia. Both POMC and PC1 mRNA expression in the pars intermedia were significantly increased in the autumn (POMC, $p<0.01$; PC1, $p<0.001$). No significant effect of season on the expression of PC2 mRNA was detected. WS- Winter solstice, SE- Spring equinox, SS- Summer solstice, AE- Autumn equinox.

Figure 4.4 Representative sections of the pars intermedia taken (SS group) subjected to in situ hybridisation with POMC (left), PC1 (middle) and PC2 (right) antisense (AS) and sense (S) cRNA probes and exposed for 2 days (POMC) or six weeks (PC1 and PC2). POMC, PC1 and PC2 mRNA are expressed in the pars intermedia but not the pars nervosa. Light field (LF) photomicrographs are also shown.


AS
LF
S
POMC
PC1
PC2
200 μm

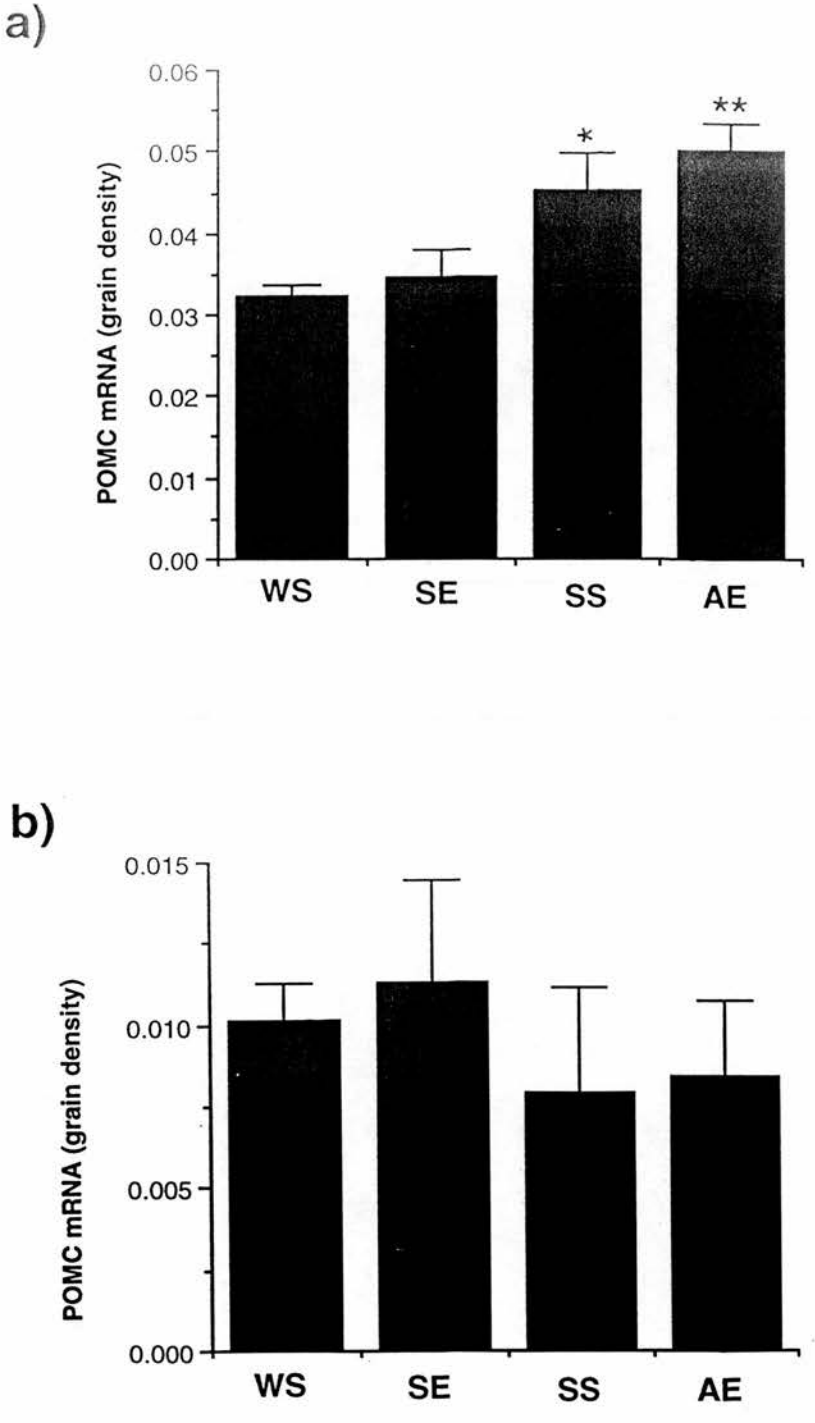


Figure 4.5 Semi-quantitative analysis of POMC in situ hybridisation in the pars intermedia (a) and pars distalis (b). POMC mRNA expression in the pars intermedia was significantly increased (by approximately 50%) from winter to autumn ($p<0.01$, ANOVA). POMC mRNA expression in the pars distalis was not affected by season. WS- Winter solstice, SE- Spring equinox, SS- Summer solstice, AE- Autumn equinox.

Figure 4.6 Representative sections of the pars distalis (SS group) subjected to in situ hybridisation with POMC (left), PC1 (middle) and PC2 (right) antisense (AS) and sense (S) cRNA probes and exposed for three days (POMC) or six weeks (PC1 and PC2). POMC and PC1 mRNA are both expressed in a scattered population of cells in the pars distalis. PC2 mRNA was not detected in the pars distalis. Light field (LF) photomicrographs are also shown.

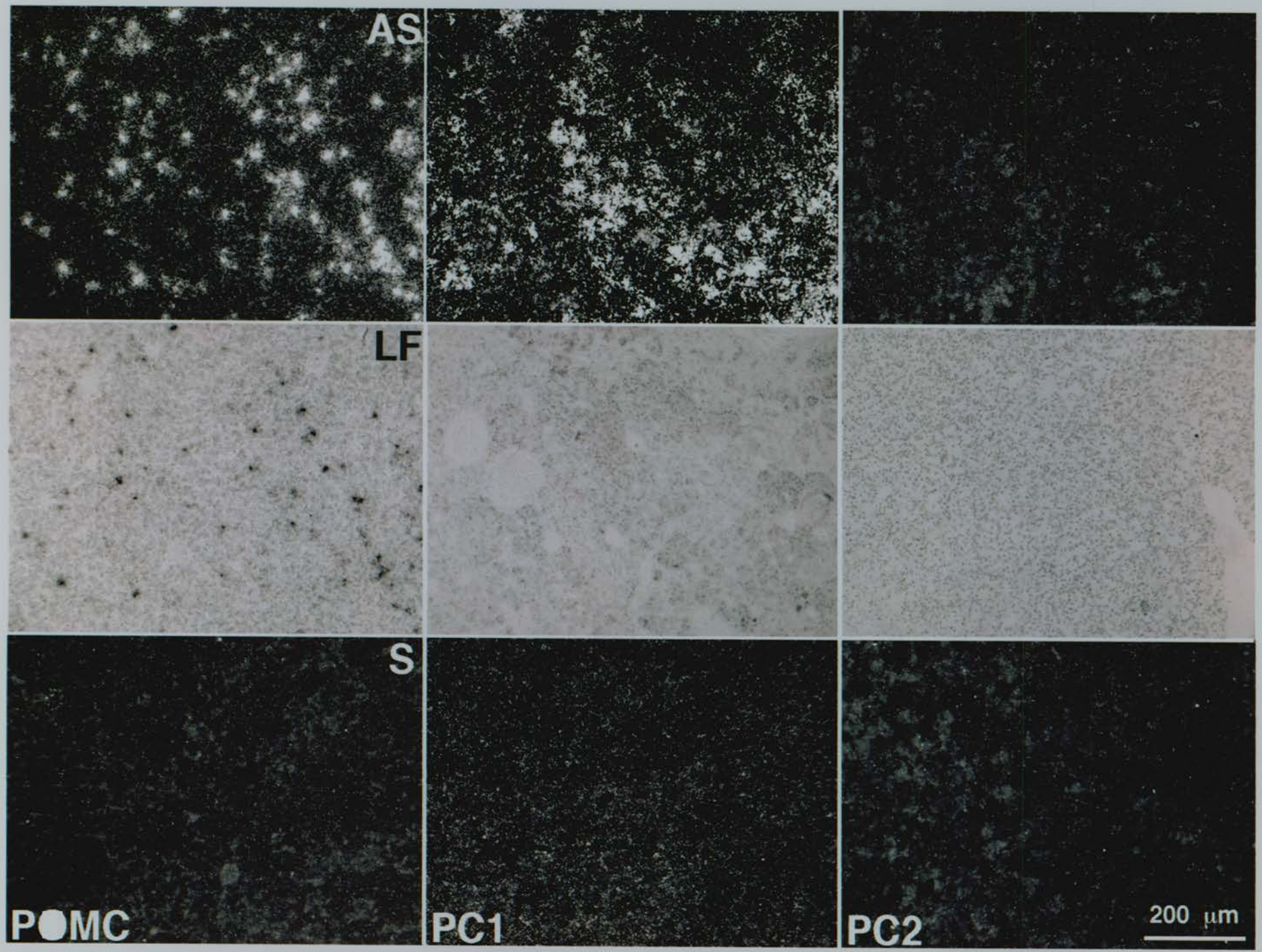

AS
LF
S
POMC
PC1
PC2
200 μm

Figure 4.7 Northern blot analysis on 5μg total RNA probed with radiolabelled PC1 cDNA. PC1 mRNA transcripts of 2.5kb are detected in the pituitary gland (Pt), the mediobasal hypothalamus (MBH), the hippocampus (Hp) and the cerebellum (Cb). Additionally, minor transcripts of 1.8, 1.4 and 0.8kb are detected in the pituitary gland. Exposed to X-OMAT LS film for 10 days.

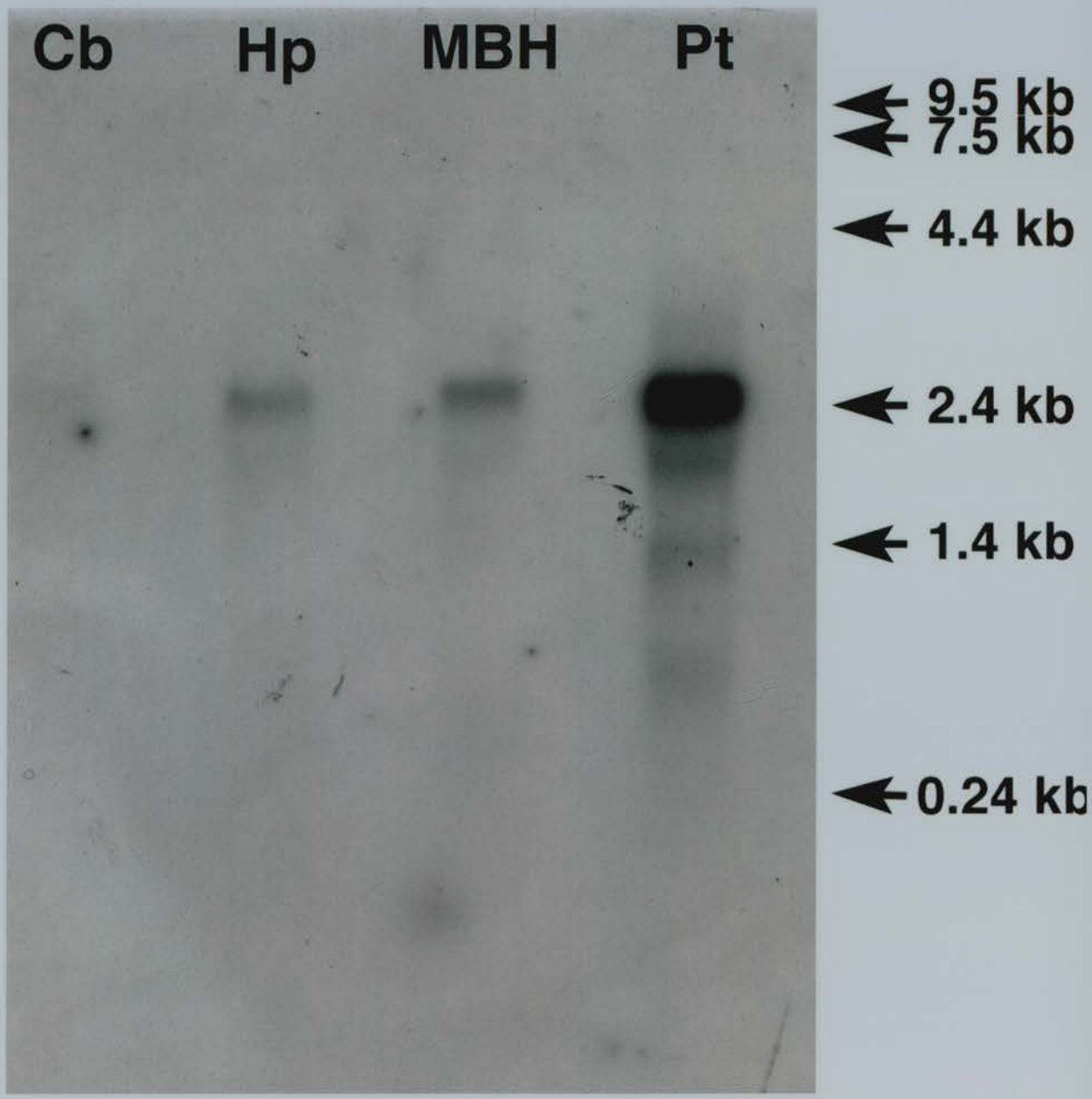
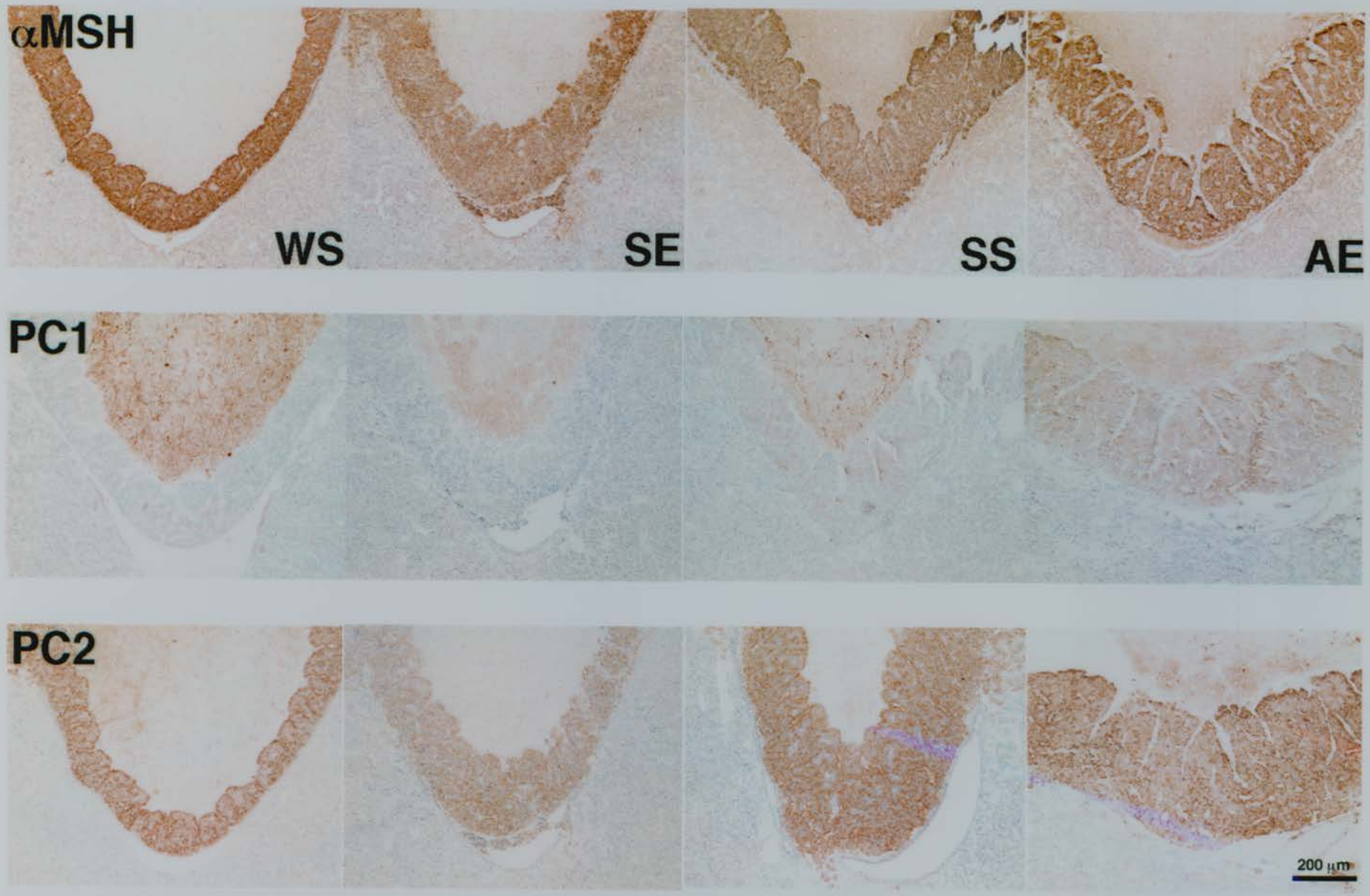

Cb
Hp
MBH
Pt
9.5 kb
7.5 kb
4.4 kb
2.4 kb
1.4 kb
0.24 kb

Figure 4.8 Representative examples of αMSH (top), PC1 (middle) and PC2 (bottom) immunocytochemical staining in the pars intermedia of Soay ewes living outdoors and killed at four points in the year (WS- Winter solstice, SE- Spring equinox, SS- Summer solstice, AE- Autumn equinox). PC1 staining intensity in the pars intermedia tended to be increased in summer and autumn but this was not quite statistically significant. Both αMSH and PC2 staining intensity in the pars intermedia were unaffected by season.


αMSH
WS
SE
SS
AE
PC1
PC2
200 μm

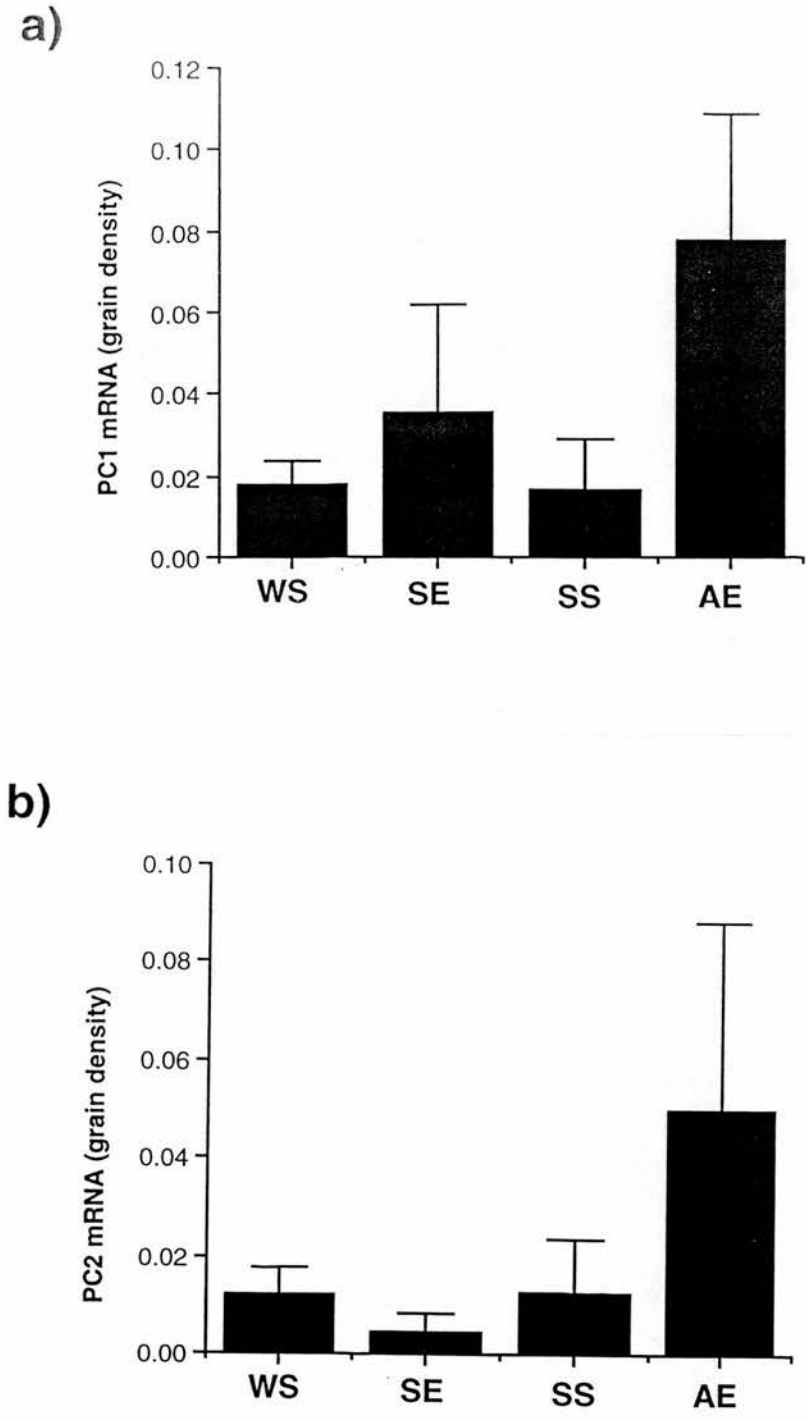
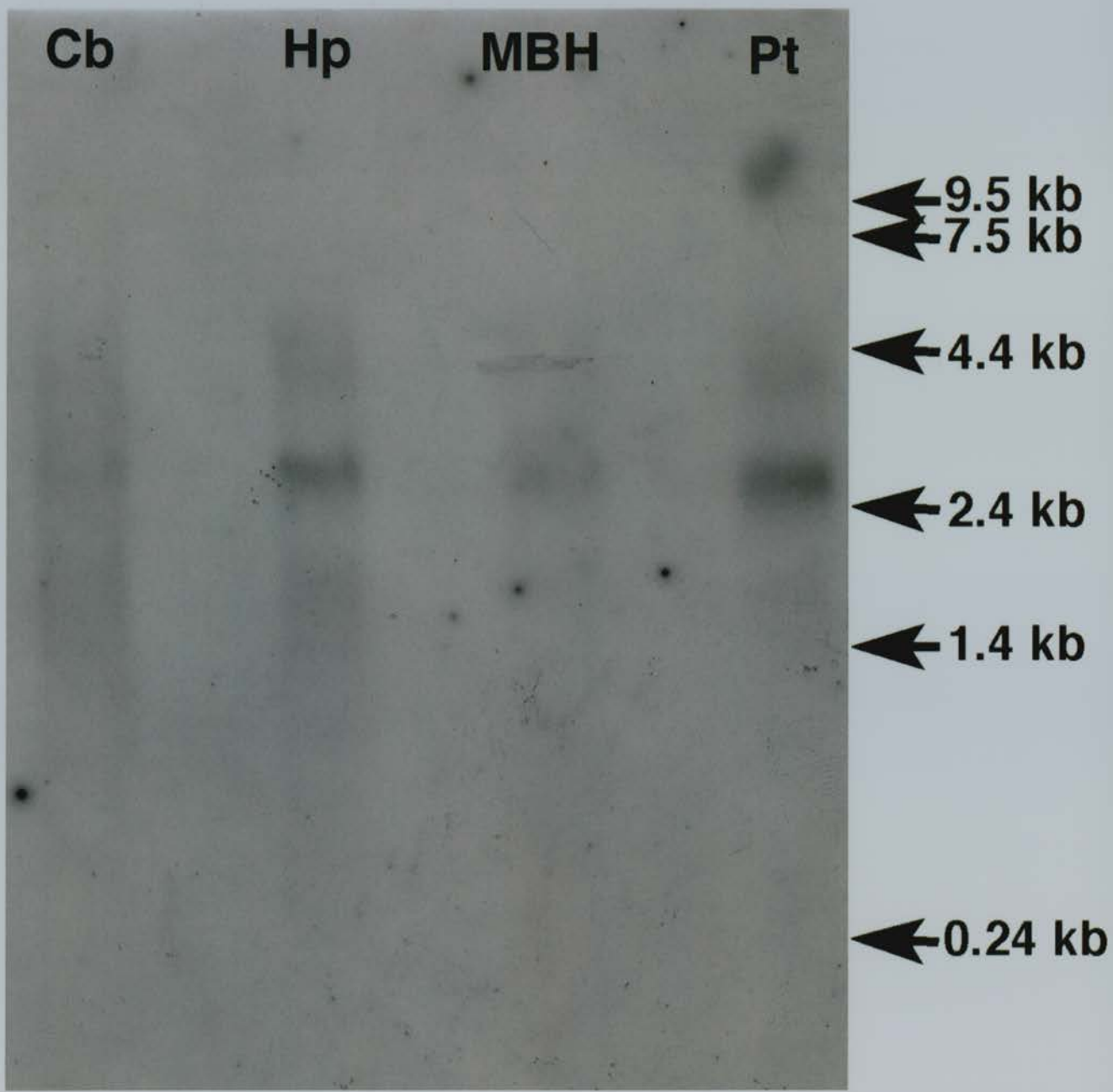


Figure 4.9 Semi-quantitative analysis of PC1 (a) and PC2 (b) in situ hybridisation in the pars intermedia. Both PC1 and PC2 mRNA expression in the pars intermedia tended to be increased in the autumn but were highly variable between individuals and were not statistically affected by season. WS- Winter solstice, SE- Spring equinox, SS- Summer solstice, AE- Autumn equinox.

Figure 4.10 Northern blot analysis on 5μg total RNA probed with radiolabelled PC2 cDNA. PC2 mRNA transcripts of 2.7kB are detected in the pituitary gland (Pt), the mediobasal hypothalamus (MBH) and the hippocampus (Hp). PC2 mRNA was not detected in the cerebellum (Cb). Exposed to X-OMAT LS film for 10 days.


Cb
Hp
MBH
Pt
9.5 kb
7.5 kb
4.4 kb
2.4 kb
1.4 kb
0.24 kb

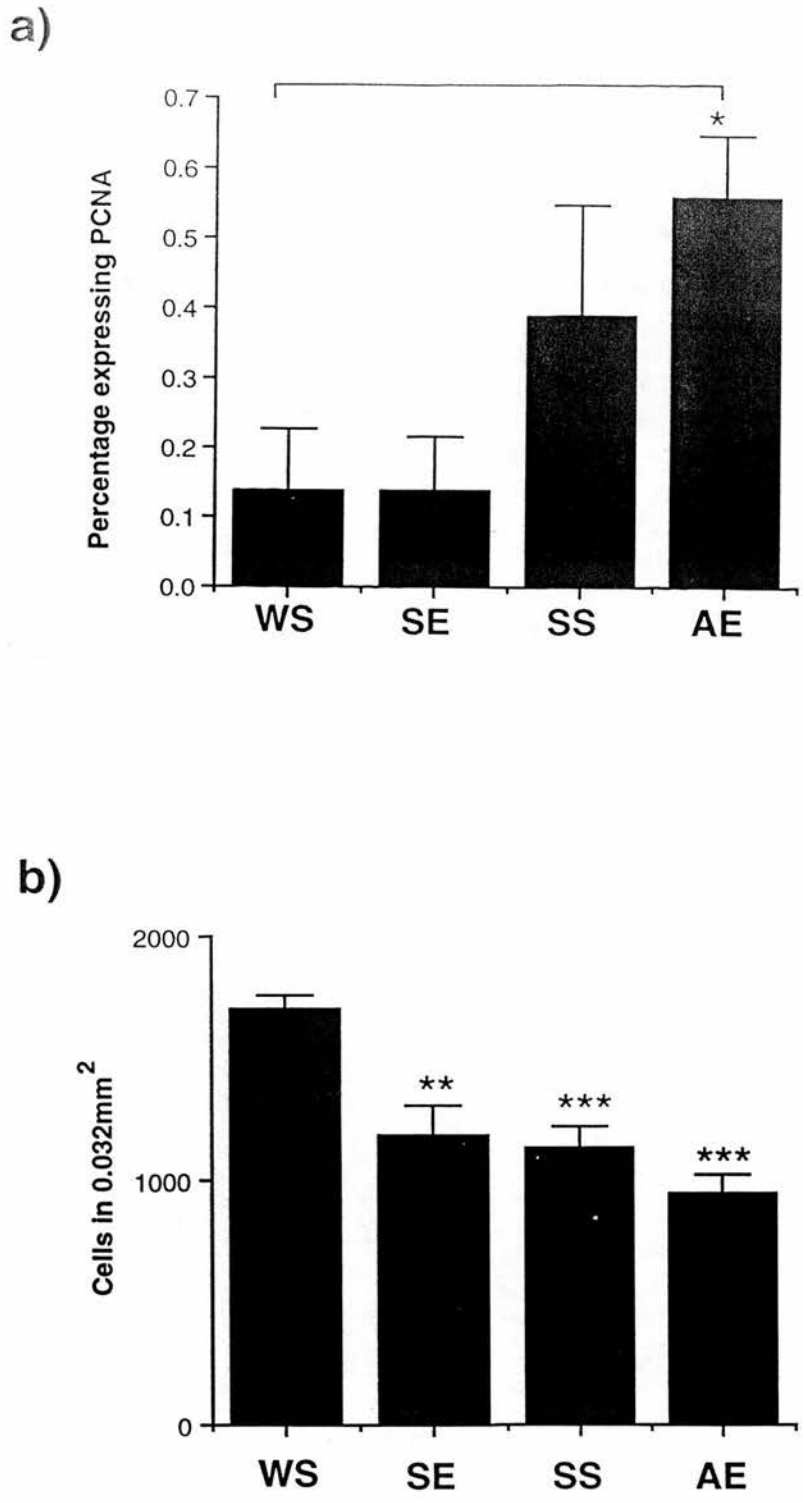


Figure 4.11 Histological analysis of melanotroph proliferation and density in the pars intermedia. a) PCNA expression increased from winter to autumn ($p<0.05$, ANOVA) indicating increased cellular proliferation. b) Melanotroph cell density decreased siginificantly over time from winter to autumn ($p<0.0001$, ANOVA) indicating pars intermedia hypertrophy over the experiment. WS- Winter solstice, SE- Spring equinox, SS- Summer solstice, AE- Autumn equinox.

Chapter 5

Photoperiodic and hypothalamic regulation of pars intermedia POMC expression and processing

5.1 Introduction

In the last chapter, evidence was presented that the seasonal cycle in circulating αMSH (Lincoln and Baker, 1995) is associated with corresponding seasonal changes in the expression of POMC its endoproteolytic processing enzymes, PC1 and PC2, within the pars intermedia. Photoperiod is the principle environmental cue dictating the timing of this cycle (Lincoln and Baker, 1995). This effect of photoperiod is believed to be mediated by melatonin through its action within the hypothalamus (Lincoln and Maeda, 1992a). The aims of the current experiment were to measure the effect of the photoperiod and the role of the hypothalamus on the expression of POMC mRNA and the mRNAs of the enzymes involved in the endoproteolytic processing of the POMC peptide in the pars intermedia of the Soay sheep. This was investigated in animals housed under an artificial light cycle designed to drive the cycle in αMSH secretion and in animals in which the hypothalamus was surgically disconnected from the pituitary gland. The prediction was that the POMC, PC1 and PC2 mRNA expression would be enhanced under short days compared to long days and that this effect would be ablated following hypothalamo-pituitary disconnection.

5.2 Experimental procedures

5.2.1 Animal treatments and collection of tissues

The animals used were adult Soay rams housed indoors under an artificial lighting regime of alternating 16-weekly periods of long (LD; 16L:8D) and short (SD; 8L:16D) days. Animals were housed in individual pens with visual and tactile contact between neighbours and were fed on a constant diet of dried grass nuts with hay and water *ad libitum* (section 2.1.1).

The animals used in this study were intact and long term HPD Soay rams (table 5.1). The HPD procedure was performed by Iain Clarke and Gerald Lincoln approximately three years before killing as described by Clarke et al (1983). The HPD surgical procedure removed the nerve terminals at the median eminence as well as the axons innervating the pars nervosa while sparing the superior arterial system supplying blood to the pituitary gland. A foil barrier was inserted between

the hypothalamus and the pituitary gland to prevent the re-innervation of the pituitary gland.

The animals were killed at seven weeks into the long or short day treatment (section 2.1.2). Immediately after death, the pituitary glands were separated, weighed and dissected into rostral and caudal portions (section 2.3.1). Tissues from the rostral part were promptly fixed for five hours in Bouins reagent and processed for histology as described in section 2.4. The remaining tissue from the caudal part was dissected into pars intermedia/pars nervosa and pars distalis, snap frozen and stored at -70°C until RNA extraction (section 2.3). These tissues were utilised in RNase protection assays, in situ hybridisation and immunocytochemistry.

Animal	Body weight (Kg)	Pituitary Wt (mg)	Treatment/ Photoperiod
S18	41	539	Intact/SD
S19	45	740	Intact/SD
S20	42	807	Intact/SD
S21	45	519	Intact/SD
S30	40	601	Intact/SD
S22	40	663	Intact/LD
S24	33	-	Intact/LD
S25	34	691	Intact/LD
S26	33	658	Intact/LD
S74	36	392	HPD/SD
S75	42	356	HPD/SD
S77	50	487	HPD/SD
S66	39	266	HPD/LD
S67	40	317	HPD/LD
S73	39	550	HPD/LD

Table 5.1 Animal data at time of culling.

5.2.2 RNase protection assay

RNase protection assays were carried out for POMC, PC1 and PC2 on 5μg total pars intermedia RNA from intact LD and SD groups using cRNA probes generated from the subcloned ovine cDNA sequences described in detail in section 2.8. The size of the protected fragments was assessed by comparison with RNA size standards (Ambion). All RNase protection assays protected partial transcipts of the expected size. The relative optical density of POMC, PC1 and PC2 RNA and 18S ribosomal standard were determined on a phosphorimager (Molecular Dynamics) and the 18S ribosomal standard was used to correct for loading variations in the RNA between treatments. Differences between the relative density in the different treatment groups were evaluated using the unpaired student t-test. Where the

standard deviations differed between treatments, the natural logarithms of data were used for parametric comparisons.

5.2.3 In situ hybridisation

In situ hybridisation was carried out for POMC, PC1 and PC2 mRNA (section 2.12) on tissues from all animals. In situ hybridisation employed the same ovine cDNA sequences as RNase protection assay (section 2.8). The tissue sections were exposed to photographic emulsions for a period ranging from 1-2 days (POMC) to six weeks (PC1 and PC2; section 2.11.6). Semi-quantitative analyses were undertaken using computer-aided image analysis (section 2.11). Analysis of variance was applied to grain density measurements obtained by image analysis between treatments. As with RNase protection assay, transformed data was used where standard deviations differed between treatments.

5.2.4 Immunocytochemistry

Immunocytochemical staining for αMSH, ACTH, PC1 and PC2 was carried out on 3μm coronal sections of the pituitary gland of all animals (section 2.5.4). Where semi-quantitative analysis of immunocytochemical staining intensity was made, sections were examined without prior knowledge of treatment group and staining intensity was graded from zero (antigen not detectable) to five (heavy immunostaining).

To measure pars intermedia cellular proliferative activity, immunocytochemistry for the proliferation marker PCNA was undertaken (section 2.5.4). Percentage positive staining and cell density measurements were carried out simultaneously by counting total and positively-staining nuclei within ten graticule units (Graticules Ltd, Tonbridge, UK) in ten different areas of the tissue (total area in which cells were counted was $0.032mm^2$). Counting of cells was made on slides without prior knowledge of treatment group and comparisons between groups were made using ANOVA. The cross-sectional area of the pars intermedia, pars distalis and pars nervosa were measured using an image analysis program (Proplus software).

5.3 Results

5.3.1 Animals

In intact animals, the body weight was significantly greater in the SD compared with the LD treatment group (Intact SD, 42.6±1.0, Intact LD, 35.0±1.7kg;

$p<0.01$) at the time of killing. The body weight in the HPD animals was not affected by photoperiod and was not different from the mean body weights of the intact animals (HPD SD, 42.7±4.1, HPD LD, 39.3±0.3kg).

5.3.2 POMC expression

In the intact animals, pars intermedia POMC mRNA expression, as measured by RNase protection assay, tended to be higher in the intact SD group compared to intact LD group, although this effect was not quite statistically significant (Figures 5.1 and 5.2a; Intact SD, 0.552±0.169, Intact LD, 0.146±0.020; $p<0.1$). Similarly, in situ hybridisation indicated a similar trend in POMC mRNA expression related to the photoperiod in the pars intermedia (Figures 5.3 and 5.4a; Intact SD, 1.062±0.063, Intact LD, 0.599±0.216; $p<0.1$). However, in the HPD rams, photoperiod had no effect on POMC mRNA expression in the pars intermedia (Figures 5.7 and 5.8; HPD SD, 0.741±0.143, HPD LD, 0.610±0.135). POMC mRNA expression in HPD animals was similar to that in intact animals exposed to LD treatment.

5.3.3 PC1 expression

Pars intermedia PC1 mRNA expression was significantly increased in intact SD compared to intact LD animals as measured by in situ hybridisation (Figures 5.3 and 5.4b; Intact SD, 0.0202±0.0098, Intact LD, 0.0007±0.0004; $p<0.05$). RNase protection assay also indicated a similar effect of photoperiod on the expression of PC1 in the pars intermedia of intact animals (Figure 5.1 and 5.2b; Intact SD, 1.40±0.29, Intact LD, 0.52±0.11; $p<0.05$). PC1 peptide immunocytochemical staining was detectable in the pars intermedia of all animals with increased staining in intact SD compared to intact LD animals (Figure 5.5; Intact SD, 2.4±0.7, Intact LD, 1.0±0.0). PC1 immunocytochemical staining was also detected in the pars distalis of all animals with no effect of photoperiod (Intact SD, 2.2±0.7, Intact LD, 3.0±0.4).

In the HPD animals pars intermedia PC1 mRNA expression was unaffected by photoperiod as measured by in situ hybridisation (Figures 5.7 and 5.8b, HPD SD, 0.0005±0.0005, HPD LD, 0.0009±0.0005). However, PC1 mRNA was reduced in HPD animals compared to intact SD animals (Figures 5.7 and 5.8b Intact SD, 0.0202±0.0098, Intact LD, 0.0007±0.0004, HPD SD, 0.0005±0.0005, HPD LD, 0.0009±0.0005; $p< 0.05$). PC1 peptide immunocytochemical staining was detected in the pars intermedia of all HPD animals with no difference in staining intensity with photoperiod or in comparison with intact animals (Figure 5.9, Intact SD, 2.4±1.0, Intact LD, 1.0±0.0, HPD SD, 3.0±0.6, HPD LD,

2.0±0.6). In the pars nervosa PC1 immunocytochemical staining was reduced in HPD animals compared to intact controls (Intact SD, 3.2±0.4, Intact LD, 3.8±0.6, HPD SD, 1.7±0.9, HPD LD, 0.7±0.7).

5.3.4 PC2 expression

No effect of photoperiod on pars intermedia PC2 mRNA expression was detectable by RNase protection assay (Figures 5.1 and 5.2c; Intact SD, 1.08±0.79, Intact LD, 0.16±0.02) or by in situ hybridisation (Figures 5.3 and 5.4c; Intact SD, 0.0328±0.0185, Intact LD, 0.0059±0.0039), although values obtained by both techniques indicated that PC2 mRNA expression tended to be higher in SD than in LD animals.

PC2 peptide was detectable in the pars intermedia, pars nervosa and weakly in the pars distalis of all animals and was not affected by treatment in either the pars intermedia (Figure 5.5; Intact SD, 2.2±0.4, Intact LD, 3.0±0.0) or pars distalis (Intact SD, 0.4±0.2, Intact LD, 0.3±0.3).

In HPD animals, pars intermedia PC2 mRNA expression was unaffected by photoperiod (Figures 5.7 and 5.8c; HPD SD, 0.0178±0.0097, HPD LD, 0.0036±0.0019). PC2 mRNA expression was also unaltered in HPD compared to intact animals. Pars intermedia PC2 immunocytochemical staining was not affected by photoperiod or HPD (Figure 5.9; Intact SD, 2.2±0.4, Intact LD, 3.0±0.0, HPD SD, 3.7±0.3, HPD LD, 3.0±0.6). PC2 immunoreactivity was, however, reduced in the pars nervosa of HPD compared to intact animals (Figure 5.9; Intact SD, 2.0±0.3, Intact LD, 2.3±0.3, HPD, 1.2±0.3).

5.3.5 Pars intermedia αMSH and ACTH expression

Strong αMSH-immunocytochemical staining was detected in the pars intermedia in all animals (Figures 5.5 and 5.9). The staining intensity in the pars intermedia did not vary with photoperiod in either intact or HPD animals (Figures 5.5 and 5.9; Intact SD, 3.0±0.3, Intact LD 3.8±0.3, HPD SD, 3.0±1.0, HPD LD, 2.6±0.3). Localised low levels of αMSH immunocytochemical staining were also detected in the pars distalis and were unaffected by photoperiod (Intact SD, 1.2±0.8, Intact LD, 1.0±0.0). Pars distalis αMSH staining intensity was, however, increased in HPD compared to intact LD animals (Figures 5.5 and 5.9; Intact SD, 1.2±0.8, Intact LD, 1.0±0.0, HPD SD, 1.7±0.3, HPD LD, 2.7±0.3). The pattern of staining for ACTH was comparable to that of αMSH in the pars intermedia whereas in the pars distalis ACTH immunocytochemical staining was localised to a subpopulation of cells and where present was more intense than in the pars intermedia. ACTH immunocytochemical staining intensity was unaffected by

photoperiod and HPD in both tissues (Pars intermedia; Intact SD, 3.2±0.4, Intact LD, 3.0±0.4, HPD SD, 3.3±0.3, HPD LD, 2.3±0.3; Pars distalis; Intact SD, 3.4±0.2, Intact LD, 3.8±0.3, HPD SD, 3.5±0.5, HPD LD, 4.0±0.0).

5.3.6 Pars intermedia cell proliferation and morphology

Cells expressing PCNA were infrequent and were only detected in intact LD animals. The low numbers of PCNA expressing cells precluded the detection of an effect of photoperiod on cell proliferation in the pars intermedia (Intact SD, 0.00, Intact LD, 0.09±0.04%, Figure 5.6a). Cell density was also unaffected by photoperiod or HPD (Intact SD, 1059±53, Intact LD, 1119±47 cells in 0.032mm^2, Figure 5.6b).

5.4 Discussion

The data presented in this chapter provides tentative support for the prediction that POMC, PC1 and PC2 mRNA expression is enhanced in the pars intermedia under short days and that this effect is blocked by HPD. However, only the expression of PC1 mRNA was significantly increased in the intact Soay rams under short days, while POMC and PC2 mRNA expression were increased, but this change was not statistically significant. These relatively minor changes in mRNA expression are in contrast with the predictably large changes in the circulating concentrations of αMSH observed under similar conditions (Lincoln and Baker, 1995). This difference in mRNA expression and plasma peptide concentrations may indicate that relatively small changes in gene expression result in relatively large changes in peptide secretion if changes in the expresion of these genes have a compounding or additive effect. Alternatively, this observed difference may be largely due to the secretion of the αMSH peptide, unrelated to the expression of these genes.

The immunocytochemical studies confirmed the presence of αMSH, PC1 and PC2 in the pars intermedia. Semi-quantitation of the immunocytochemical staining revealed a significant increase in PC1 protein under short days without a corresponding change in PC2. This tends to support the view that increased production of αMSH is associated with increased expression of at least one of the POMC processing enzymes. However, the cellular content of the enzyme may not reflect the rate of secretion of its product or its functional activity. The intensity of immunocytochemical staining of the peptide product itself does not reflect the pattern of its secretion, since there were no observed changes in the cellular content of αMSH in the pars intermedia under short days when large difference in circulating peptide would be predicted. Indeed, in the Siberian hamster,

hypersecretion of αMSH results in a decrease in the cellular content of the protein when release outstrips synthesis (Logan and Weatherhead, 1980).

In the HPD rams, there was no effect of photoperiod on the expression of POMC, PC1 and PC2. This is consistent with previous observations that pituitary denervation blocks the photoperiodic control of the cycle in circulating αMSH concentrations (Lincoln and Richardson, 1998) and supports the view that the hypothalamus mediates the effect of photoperiod on the pars intermedia POMC system. Furthermore, there is evidence in the short-tailed weasel, that the seasonally-induced changes in pelage colour, likely to be influenced by αMSH, are blocked where the pituitary gland is isolated from the hypothalamus by autotransplantation to the kidney capsule (Rust and Meyer, 1968).

The HPD animals used in this study had received the surgery approximately three years earlier and showed signs of permanent pituitary disconnection. This included polyurea due to the atrophy of the pars nervosa and loss of PC1 and PC2 immunoreactivity in the pars nervosa in the HPD animals. The level of expression of PC1 in the pars intermedia was decreased in HPD animals compared to intact animals exposed to short days. Moreover, POMC and PC2 expression followed a similar trend in HPD animals. Previous studies have reported that the expression of POMC mRNA and content of αMSH were markedly increased following the HPD procedure (Clarke *et al.*, 1986). This inconsistency is largely explained by the timing of the HPD operation. In the current study, the animals were chronically disconnected, having received the operation approximately three years earlier, while the study by Clarke et al was on short term HPD animals. Circulating αMSH concentrations are reported to increase immediately following HPD and then decline progressively in the long term (Lincoln and Richardson, 1998). These changes appear to be reflected by long term parallel changes in POMC gene expression. Thus, a functional hypothalamus is required to maintain normal secretory activity of the pars intermedia.

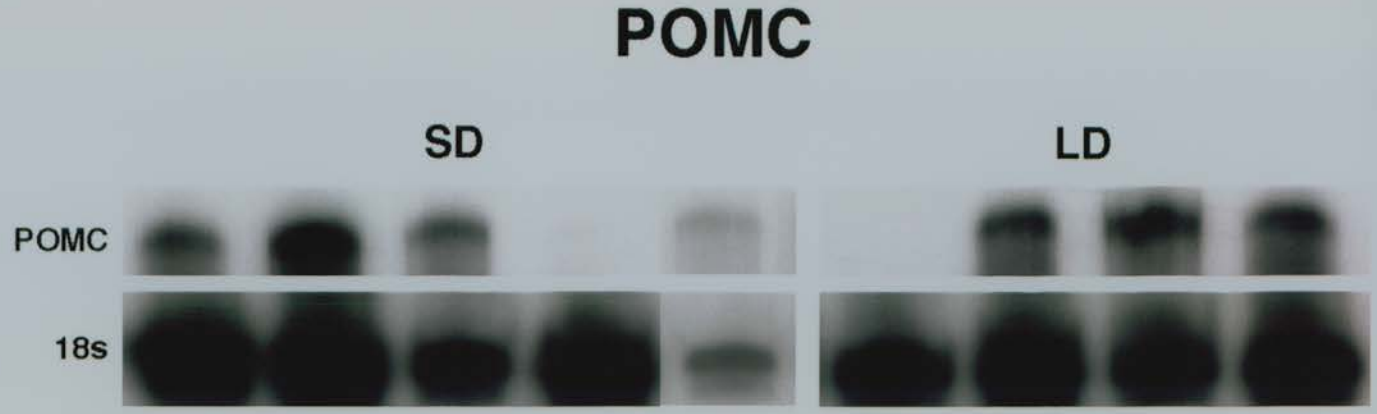
The histology revealed that the pars intermedia in the HPD rams was distorted in shape due to the regression of the pars nervosa with which it is usually closely associated. The regression of the pars nervosa was associated with the loss of PC1 and PC2 immunoreactivity in this tissue, which was striking in intact animals. The pars intermedia was also enlarged in cross-sectional area in HPD animal, although it is not clear whether this is due to cellular proliferation or hypertrophy since cellular proliferation was not detectable in long term HPD animals. In the study of the short term HPD sheep, evidence was provided of a clear hypertrophy of the pars intermedia (Clarke *et al.*, 1986; Clarke *et al.*, 1983). These morphological changes appear to be the result of the removal in

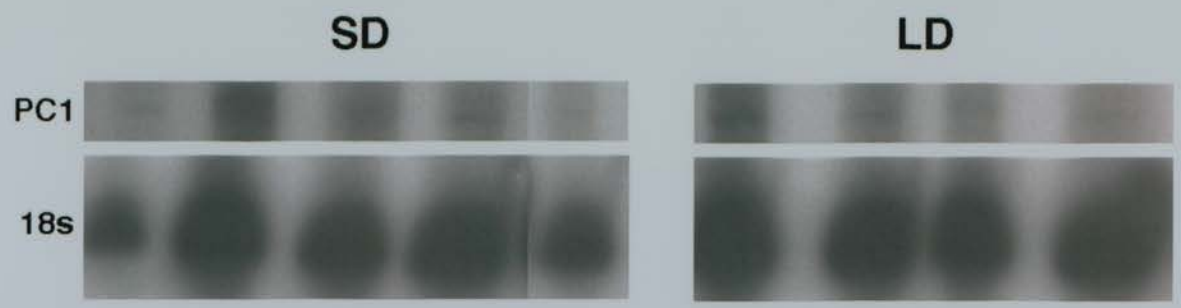
hypothalamic inhibition and further indicate that the hypothalamus regulates multiple aspects of pars intermedia function.

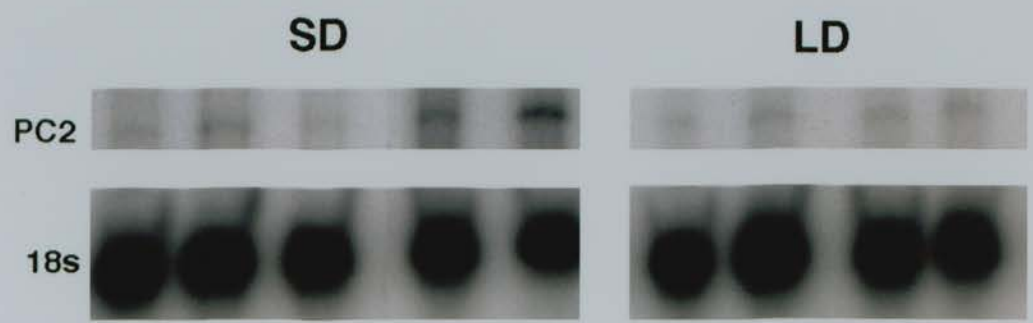
One further feature of note in the HPD animals was the increase in αMSH staining in the pars distalis, presumably associated with the corticotrophs. This may be related to the stimulatory hypothalamic control of the corticotroph resulting in a reduced release and increased storage of POMC-peptides with extended processing to yield αMSH-immunoreactive peptides. This is consistent with the observation that corticotrophs isolated from hypothalamic influence by autotransplantation to the kidney capsule contain increased amounts of αMSH. This effect in the rat is reversed by the administration of CRF (Iturriza and Ferese-Spinelli, 1991).

In summary, these results provide some support for the concept that photoperiod regulates the cyclical changes in circulating αMSH in the Soay ram by influencing the level of expression of POMC, PC1 and PC2 in the pars intermedia. Short days are stimulatory and long days are inhibitory and these effects are mediated by the hypothalamus. The potential importance of the hypothalamus as an inhibitory regulator of the pars intermedia is further investigated in the next chapter.

Figure 5.1 RNase protection assays for POMC, PC1 and PC2 mRNA in the pars intermedia of intact Soay rams exposed to 7 weeks of short day (SD) or long day (LD) photoperiods. Protected cRNA probe/mRNA band (top) and 18s (internal control) band (bottom). Exposed for 2 days (POMC) or 2 weeks (PC1 and PC2).


POMC
SD
LD
POMC
18s


PC1
SD
LD
PC1
18s


PC2
SD
LD
PC2
18s

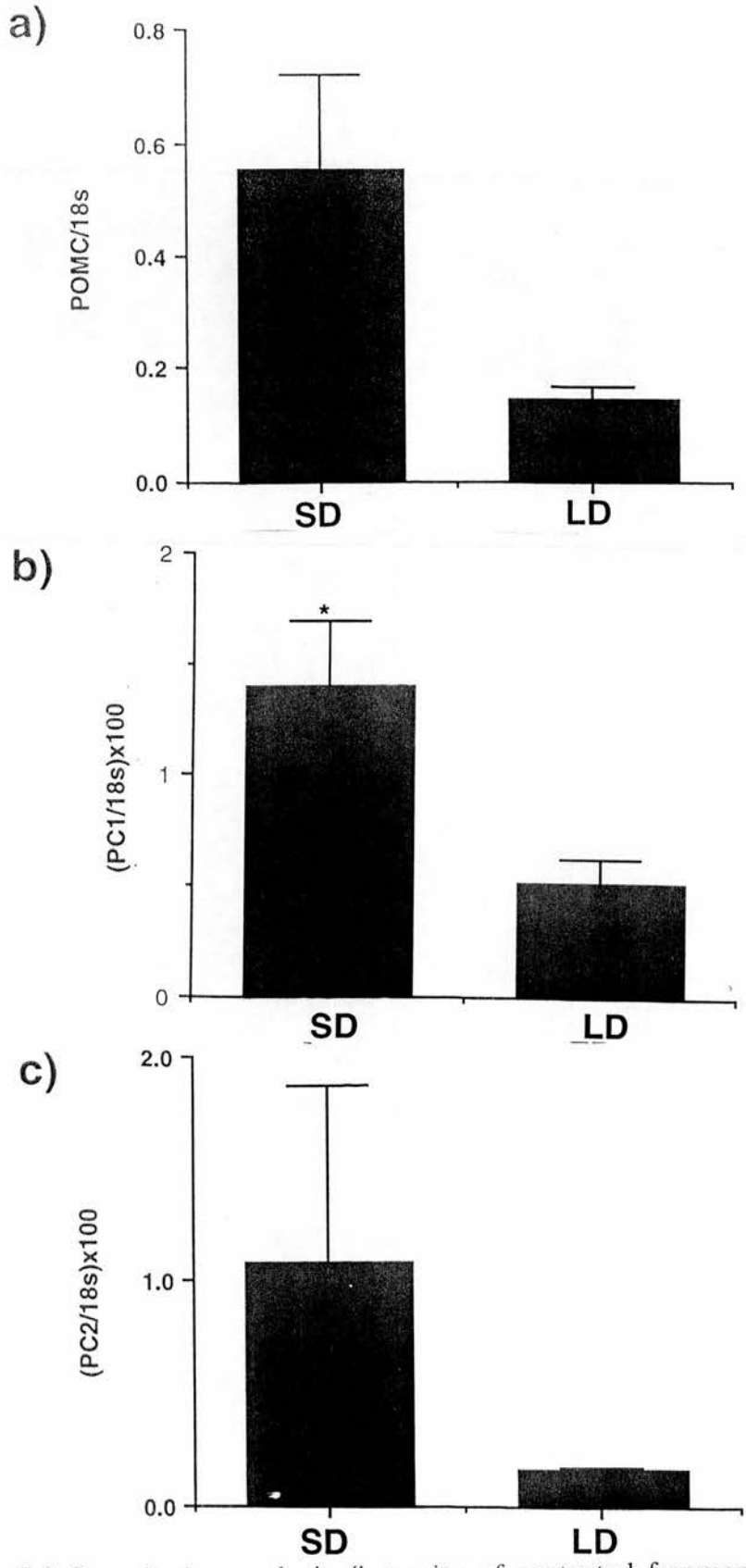
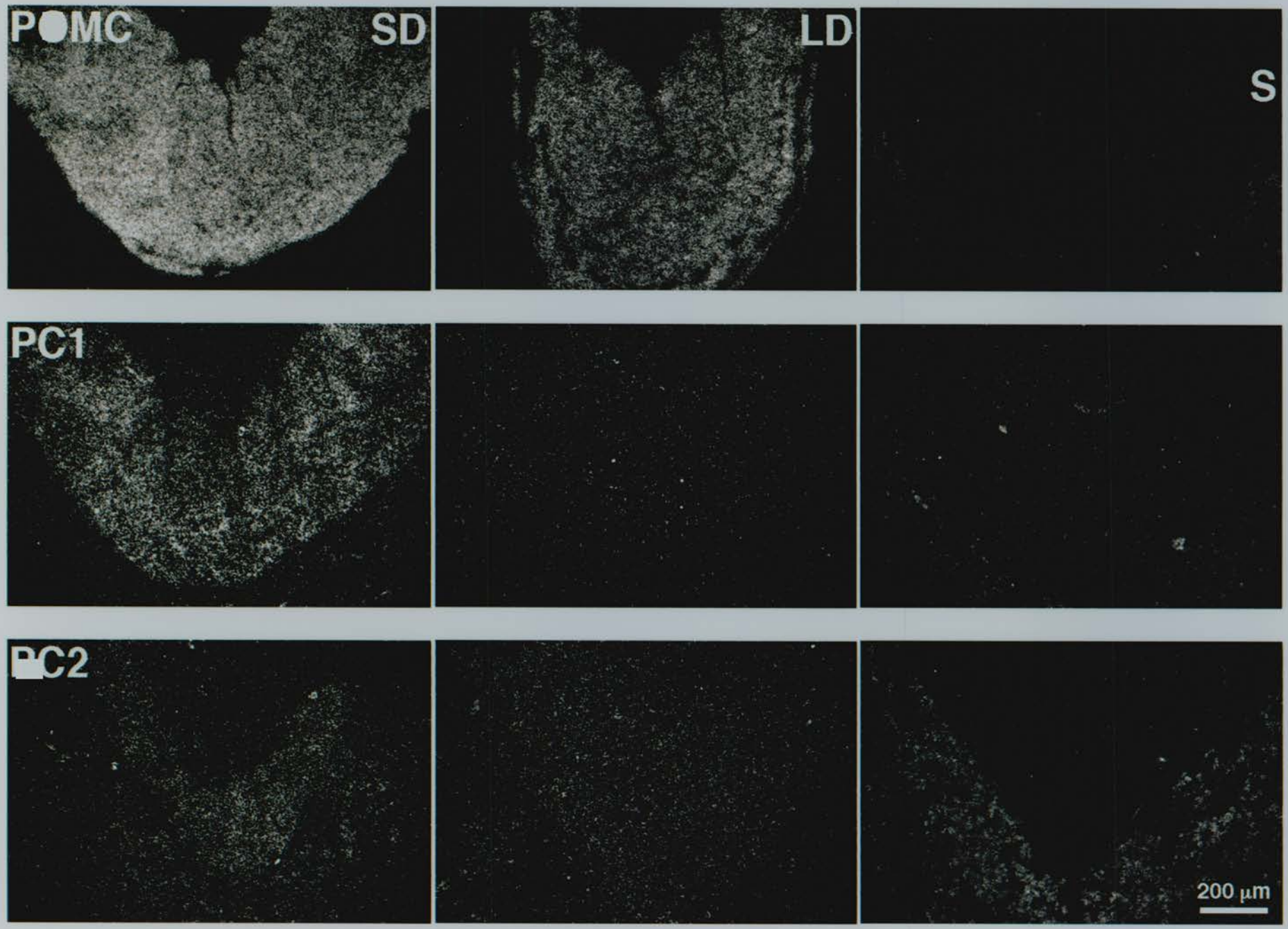


Figure 5.2 Quantitative analysis (intensity of protected fragment divided by intensity of 18s internal control) of POMC (a), PC1 (b) and PC2 (c) RNase protection assays in the pars intermedia of intact Soay rams exposed to 7 weeks of short day (SD) and long day (LD) photoperiods. The expression of all of these mRNA species tended to be decreased in animals exposed to LD photoperiod, although a statistically significant effect of treatment was only detected for PC1.

Figure 5.3 Representative sections of the pars intermedia of intact Soay rams exposed to 7 weeks of long day (LD) and short day (SD) photoperiods subjected to in situ hybridisation with POMC (top), PC1 (middle) and PC2 (bottom) antisense (AS) and sense (S) cRNA probes. Sections exposed for two days (POMC) or six weeks (PC1 and PC2).


POMC
SD
LD
S
PC1
PC2
200 μm

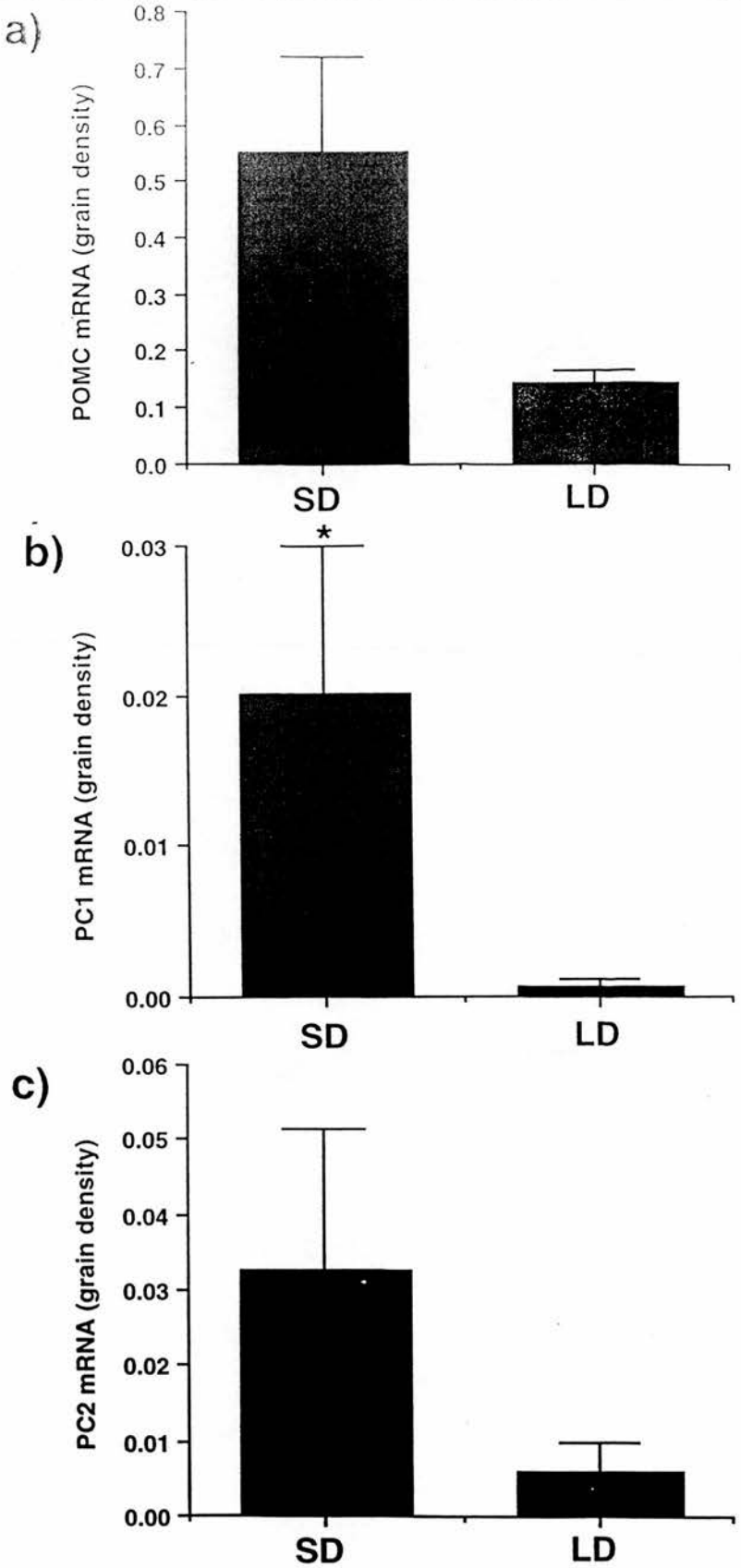
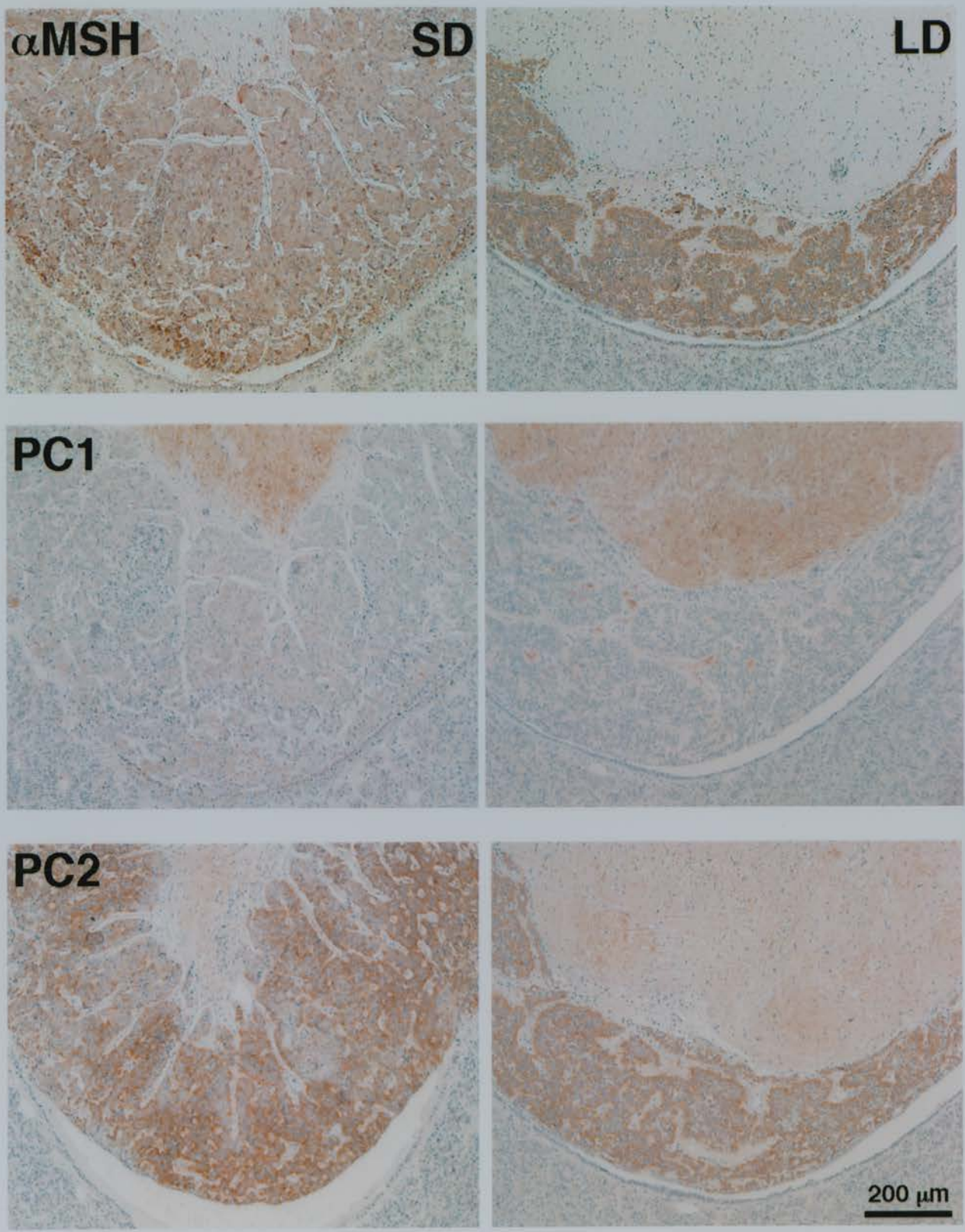


Figure 5.4 Semi-quantitative analysis of POMC (a), PC1 (b) and PC2 (c) in situ hybridisation in the pars intermedia of Soay rams exposed to 7 weeks of short day (SD) and long day (LD) photoperiods. PC1 mRNA expression was significantly reduced in LD compared to SD treatment ($p<0.05$, unpaired t-test, transformed data) No significant effect of photoperiod on either POMC or PC2 mRNA expression was detected although these tended to also be reduced in LD treatment.

Figure 5.5 Representative examples of αMSH, PC1 and PC2 immunocytochemical staining in the pars intermedia and pars nervosa of intact Soay rams exposed to short day (SD) and long day (LD) photoperiod. PC1 staining intensity in the pars intermedia tended to be higher in SD than in LD treatment, but this was not quite statistically significant ($p<0.1$, ANOVA). αMSH and PC2 staining intensity in the pars intermedia were not significantly affected by photoperiod.


αMSH
SD
LD
PC1
PC2
200 μm

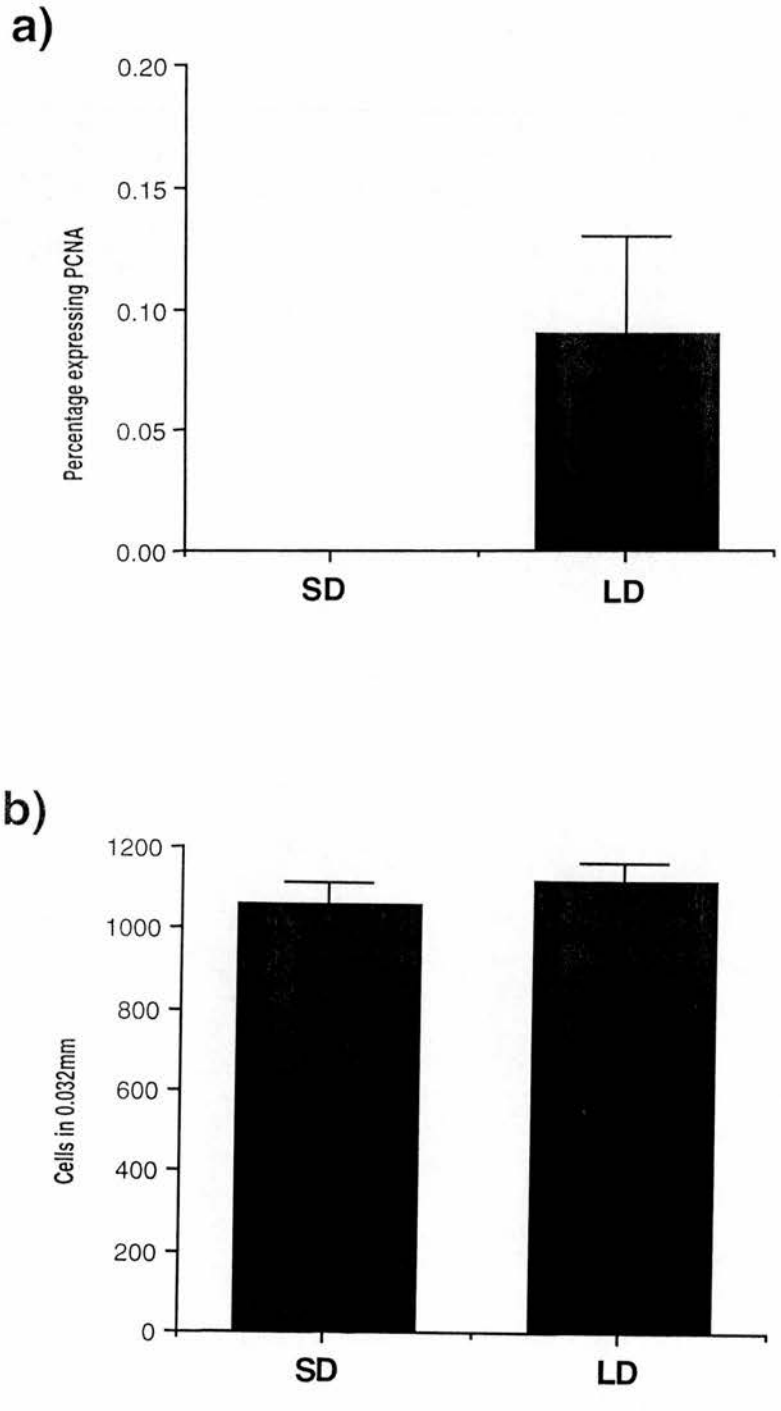
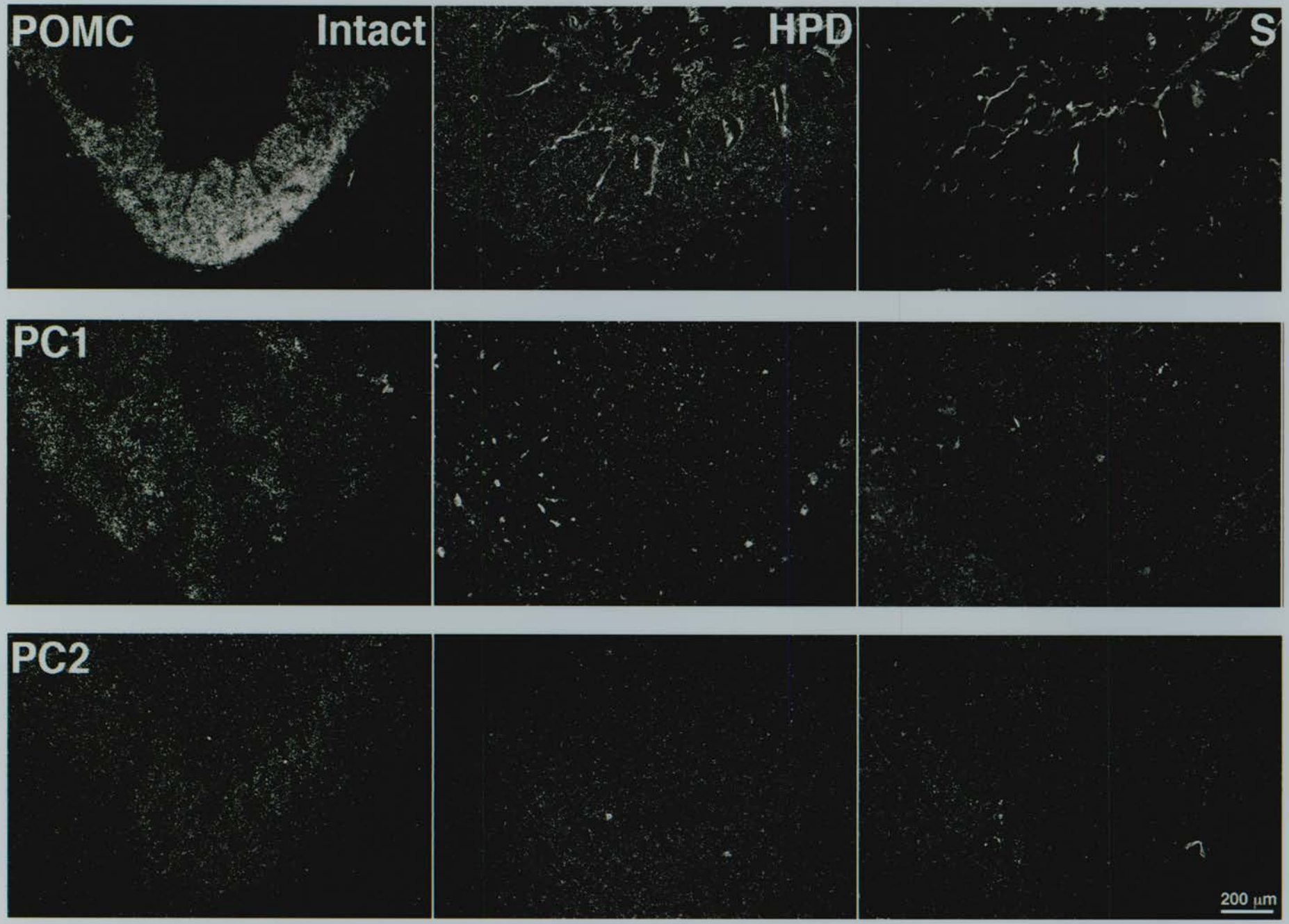


Figure 5.6 Histological analysis of melanotrophs of the pars intermedia in animals exposed to seven weeks of artificial short (SD) and long days (SD). a) PCNA expression was only detected at a low level in LD animals. b) Melanotroph cell density was unaffected by treatment.

Figure 5.7 Representative sections of the pars intermedia of intact SD and HPD SD Soay rams subjected to in situ hybridisation with POMC (top), PC1 (middle) and PC2 (bottom) antisense (AS) and sense (S) cRNA probes. Sections exposed for 2 days (POMC) or six weeks (PC1 and PC2).


POMC
Intact
HPD
S
PC1
PC2
200 µm

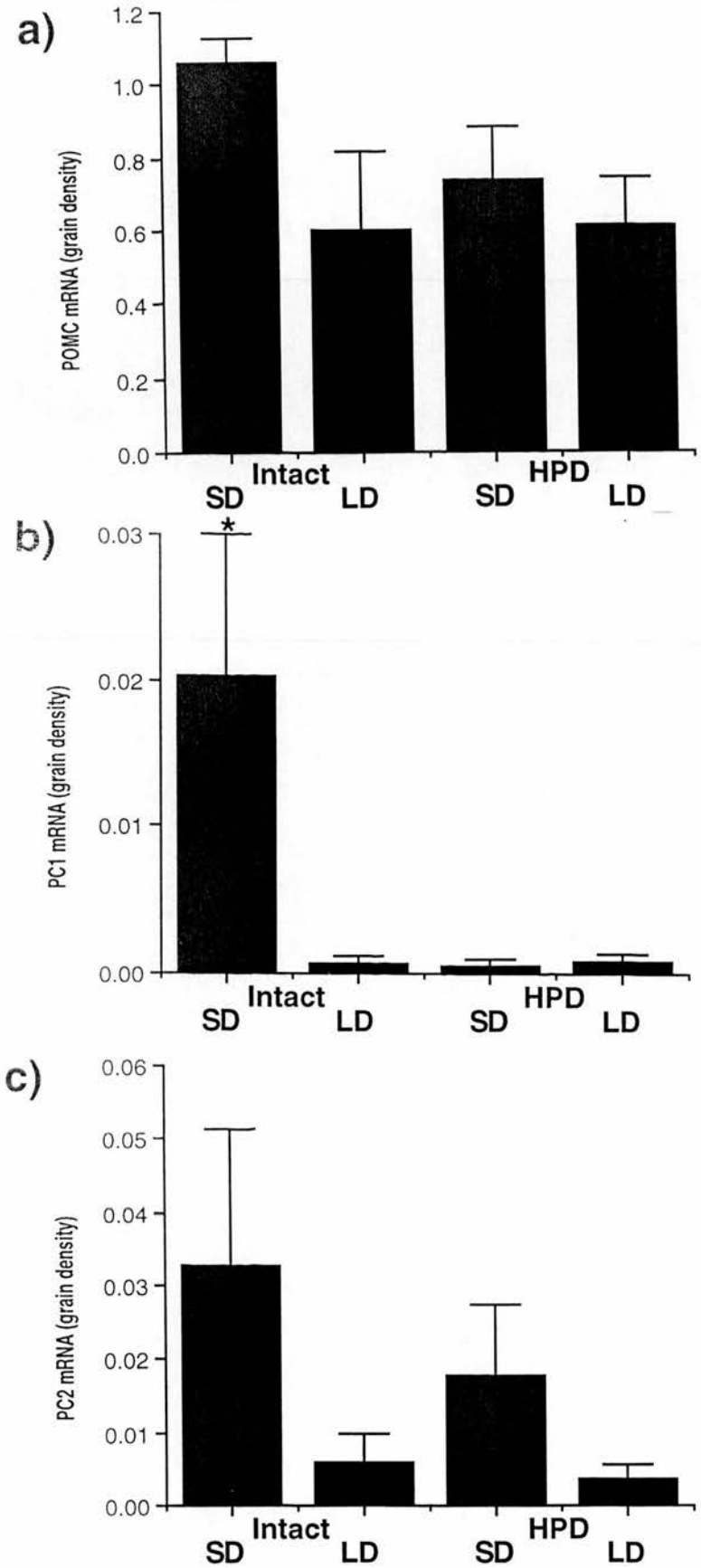
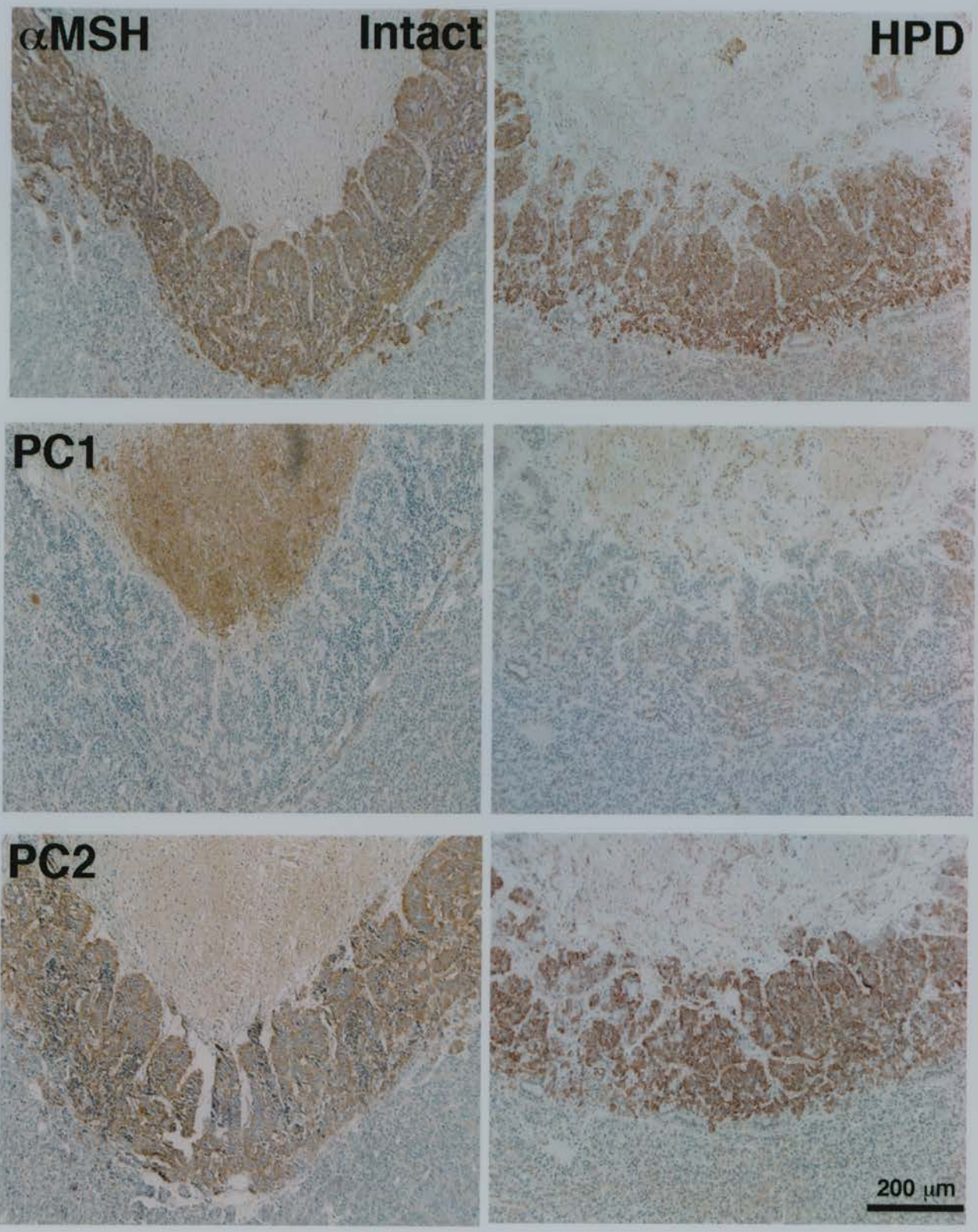


Figure 5.8 Semi-quantitative analysis of POMC (a), PC1 (b) and PC2 (c) in situ hybridisation in the pars intermedia of long term HPD and intact Soay rams exposed to 7 weeks of short day (SD), long day (LD) photoperiod. POMC mRNA expression was not significantly altered by treatment. PC1 mRNA expression in the pars intermedia was increased in the animals exposed to SD (transformed data, $p<0.05$, ANOVA) compared to LD photoperiod and HPD animals in both of which PC1 mRNA was virtually undetectable. Similarly, PC2 mRNA expression tended to be lower in both LD and HPD groups although a statistically significant effect of treatment was not detected.

Figure 5.9 Representative examples of αMSH, PC1 and PC2 immunocytochemical staining in the pars intermedia of intact and long term HPD Soay rams. αMSH, PC1 and PC2 staining intensity in the pars intermedia were not significantly affected by HPD.


αMSH
Intact
HPD
PC1
PC2
200 μm

Chapter 6.

Dopaminergic regulation of pars intermedia POMC expression and processing

6.1 Introduction

The activity of the pars intermedia is regulated through direct innervation from the hypothalamus. This hypothalamic regulation is principally inhibitory since the removal of hypothalamic influence results in increased circulating αMSH (Lincoln and Richardson, 1998). However, evidence was presented in the last chapter that suggested that in the longer term the hypothalamus is necessary for the maintenance of the secretory activity of the pars intermedia. One of the major components of this hypothalamic innervation is likely to be dopaminergic since in other species such as the rat dopamine agonists have profound inhibitory effects on the expression of POMC and its processing enzymes (Lundblad and Roberts, 1988; Oyarce *et al.*, 1996). Moreover, long term treatment with dopamine agonists also causes a reduction in the proliferative activity of the pars intermedia of the rat (Chronwall *et al.*, 1987). This study aimed to investigate the inhibitory effects of dopamine on circulating concentrations of αMSH and prolactin (the secretion of which from the pars distalis is also inhibited by dopamine), the expression of POMC, PC1 and PC2 mRNA and the cellular proliferative activity in the pars intermedia in the adult Soay ram. These parameters were measured in animals in which circulating αMSH was at its seasonal maximum, half of which received a long-acting dopamine agonist which was chronically administered over nearly six weeks.

6.2 Experimental procedures

6.2.1 Animal treatments and collection of tissues

Animals used in this study were six adult Soay rams living outdoors in Fife, Scotland (56°N), exposed to natural photoperiod in July/August when circulating αMSH is at its seasonal maximum (Ebling and Lincoln, 1987; Lincoln and Baker, 1995). Animals were maintained in grass paddocks and received supplementary feeding of commercial sheep nuts. The dopamine agonist, parlodel LA was

administered chronically (16.7mg/animal, administered five times over 39 days) to rams (n=3); three untreated rams were used as controls. When dopamine agonist was administered, blood samples were collected into heparinised tubes and plasma was obtained by centrifugation and stored at -20°C until subsequent analysis.

At the end of the experiment, animals were killed and body weight was recorded. Immediately after death, the pituitary glands were separated, weighed and dissected into rostral and caudal portions (section 2.3.1). Tissues from the rostral part were promptly fixed for five hours in Bouins reagent and processed for histology as described in section 2.4. The remaining tissue from the caudal part was dissected into pars intermedia/pars nervosa and pars distalis and snap frozen and stored at -70°C until RNA extraction (section 2.3). These tissues were utilised in RNase protection assays, in situ hybridisation and immunocytochemistry.

Animal	Body Wt (kg)	Pituitary wt (g)	Treatment
S101	36.5	768	Control
S102	28.5	354	Control
S103	33.0	532	Control
S104	31.0	473	Dopamine
S105	28.0	415	Dopamine
S106	28.0	573	Dopamine

Table 6.1 Data on animals used in studies into the dopaminergic regulation of circulating POMC-derived peptides.

6.2.2 RNase protection assay

RNase protection assays were carried out for POMC, PC1 and PC2 on 5μg total pars intermedia RNA (section 2.13) using cRNA probes generated from the subcloned ovine cDNA sequences described in detail in section 2.8. The size of the protected fragments was assessed by comparison with RNA size standards (Ambion). The relative optical density of POMC, PC1 and PC2 RNA and 18S ribosomal standard were determined on a phosphorimager and the 18S ribosomal standard was used to correct for loading variations in the RNA between treatments. Differences between the relative density in the different treatment groups were evaluated using the unpaired student t-test. Where the standard deviations differed between treatments, the natural logarithms of data were used for parametric analysis.

6.2.3 In situ hybridisation

In situ hybridisation was carried out for POMC, PC1 and PC2 (section 2.12). In situ hybridisation employed the same ovine cDNA sequences as RNase protection assay (section 2.8). The tissue sections were exposed to photographic emulsions for a period ranging from 1-2 days (POMC) to six weeks (PC1 and PC2; section 2.11.6). Semi-quantitative analyses were undertaken using computer-aided image analysis (section 2.11). The unpaired student t-test was applied to grain density measurements obtained by image analysis between treatments. As with RNase protection assay, transformed data was used where appropriate.

6.2.4 Immunocytochemistry

Immunocytochemical staining for αMSH, ACTH, PC1 and PC2 was carried out on 3μm coronal sections of the pituitary gland (Section 2.5.4). Where semi-quantitative analysis of immunocytochemical staining intensity was made, sections were examined without prior knowledge of treatment group and staining intensity was graded from zero (antigen not detectable) to five (heavy immunostaining).

To measure pars intermedia cellular proliferative activity, immunocytochemistry for the proliferation marker PCNA was undertaken (Section 2.5.4). Percentage positive staining and cell density measurements were carried out simultaneously by counting total and positively-staining nuclei within ten graticule units (Graticules Ltd, Tonbridge, UK) in ten different areas of the tissue (total area in which cells were counted was $0.032mm^2$). Counting of cells was made on slides without prior knowledge of treatment group and comparisons between groups were made using the unpaired student t-test. The cross-sectional area of the pars intermedia, pars distalis and pars nervosa was measured using an image analysis program (Proplus software).

6.2.5 Plasma hormone radioimmunoassay

Radioimmunoassays for αMSH, prolactin and FSH were carried out as described in section 2.13 on the plasma samples. Pairwise comparisons between groups were made using the student unpaired t-test. Transformed data was used for these comparisons where appropriate.

6.3 Results

6.3.1 Animals

Whole body or pituitary gland weights were unaffected by chronic treatment with dopamine agonist.

6.3.2 Plasma hormone profiles

Treatment with dopamine agonist resulted in a sustained 70% reduction in circulating αMSH concentrations compared with control rams (Control, 94.6±14.6, Dopamine agonist, 34.0±8.7pmol/litre at end (39 days) of dopamine agonist treatment; $p<0.05$; Figure 6.7a). Chronic treatment with dopamine agonist also resulted in a sustained 90% reduction in plasma prolactin (Control, 47.5±13.0, Dopamine agonist, 4.7±0.2ng/ml, $P<0.01$, transformed data, Figure 6.7b). Circulating FSH was not significantly affected by treatment with dopamine agonist (Control, 2.74±0.18, Dopamine agonist, 3.02±0.93ng/ml).

6.3.3 POMC expression

POMC RNase protection assay protected a smaller than expected POMC mRNA fragment of approximately 200bp. The intensity of this protected fragment indicated that expression of POMC mRNA was reduced in dopamine agonist-treated rams (Control, 16.92±12.55, Dopamine agonist, 0.97±0.33; $P<0.05$, Figures 6.1 and 6.2a). However, in situ hybridisation detected no significant effect of the dopamine agonist on POMC mRNA expression in the pars intermedia (Control, 0.896±0.183, Dopamine agonist, 0.600±0.141; Figures 6.3 and 6.4) or the pars distalis (Control, 0.98±0.10, Dopamine agonist, 0.91±0.33).

6.3.4 PC1 expression

In situ hybridisation indicated that pars intermedia PC1 mRNA expression was reduced by treatment with dopamine agonist (Control, 0.0805±0.0372, Dopamine agonist, 0.0027±0.0012; $p<0.05$; Figure 6.4b), although RNase protection assay detected no differences in PC1 mRNA expression between the control (2.00±2.51) and dopamine agonist-treated (0.22±0.25) rams due to high individual variation (Figures 6.1 and 6.2b).

PC1 peptide was weakly detected in the pars intermedia in both treatments by imuunocytochemistry but no effect of treatment on staining intensity was detected (Control, 1.0±0.0, Dopamine agonist, 1.3±0.3; Figure 6.5).

6.3.5 PC2 expression

RNase protection assay indicated that pars intermedia PC2 mRNA expression was significantly reduced by treatment with dopamine agonist (Control, 1.60±0.26, Dopamine agonist, 0.62±0.22; $P<0.01$, Figures 6.1 and 6.2c). However, in situ hybridisation, which detected PC2 only weakly (Figure 6.3), detected no effect of

dopamine agonist on PC2 mRNA expression in the pars intermedia (Control, 0.0053±0.0035, Dopamine agonist, 0.0046±0.0037; Figure 6.4c).

Immunocytochemistry for PC2 confirmed its presence in the pars intermedia in all animals without detecting an effect of treatment on staining intensity (Control, 2.3±0.9, Dopamine agonist, 3.7±0.3; Figure 6.5).

6.3.6 Pars intermedia αMSH and ACTH expression

Immunocytochemistry for the POMC-derived peptides, αMSH and ACTH (Figure 6.5) revealed that both peptides are present in the pars intermedia in animals from both treatment groups. The intensity of immunological staining for either peptide was not affected by treatment with dopamine agonist (αMSH; Control, 4.0±0.0, Dopamine agonist, 3.3±0.3; Figure 6.5; ACTH; Control, 1.3±0.3; Dopamine agonist, 1.7±0.3).

6.3.6 Pars intermedia cell proliferation and morphology

Immunocytochemistry for the marker of proliferation, PCNA (Figure 6.6a) suggested that pars intermedia cell proliferation was reduced in dopamine agonist-treated animals, although was just short of statistical significance (Control, 0.36±0.12, Dopamine agonist, 0.07±0.04%, $p<0.1$). Treatment with dopamine agonist resulted in hypotrophy of melanotrophs as assessed by cell density measurements using a graticule (Control 1071±131, Dopamine agonist, 1595±96 nuclei per 0.032 mm^2, $p<0.05$, unpaired t-test, Figure 6.6b).

This hypotrophy of the pars intermedia was further supported by the observation that a general reduction in the cross-sectional areas of both the pars intermedia and the pars distalis occurred in dopamine agonist-treated rams, although this was short of statisitical significance (Pars intermedia; Control, 1058205±286919, Dopmine agonist, 740641±43139μm^2; Pars distalis; Control, 15452701±1654970, Dopamine agonist, 10704795±5044597μm^2, both $p<0.2$).

6.4 Discussion

The aim of this study was to investigate whether dopamine plays a major role in the regulation of the expression of POMC, PC1 and PC2 in the pars intermedia of the Soay ram. The prediction was that the administration of dopamine agonist at the peak of the αMSH cycle would suppress the mRNA expression of POMC and its associated endoproteolytic processing enzymes. The results presented in this chapter indicate that dopamine agonist treatment caused a marked reduction in PC1 and PC2 mRNA expression but only a marginal effect for POMC. This was

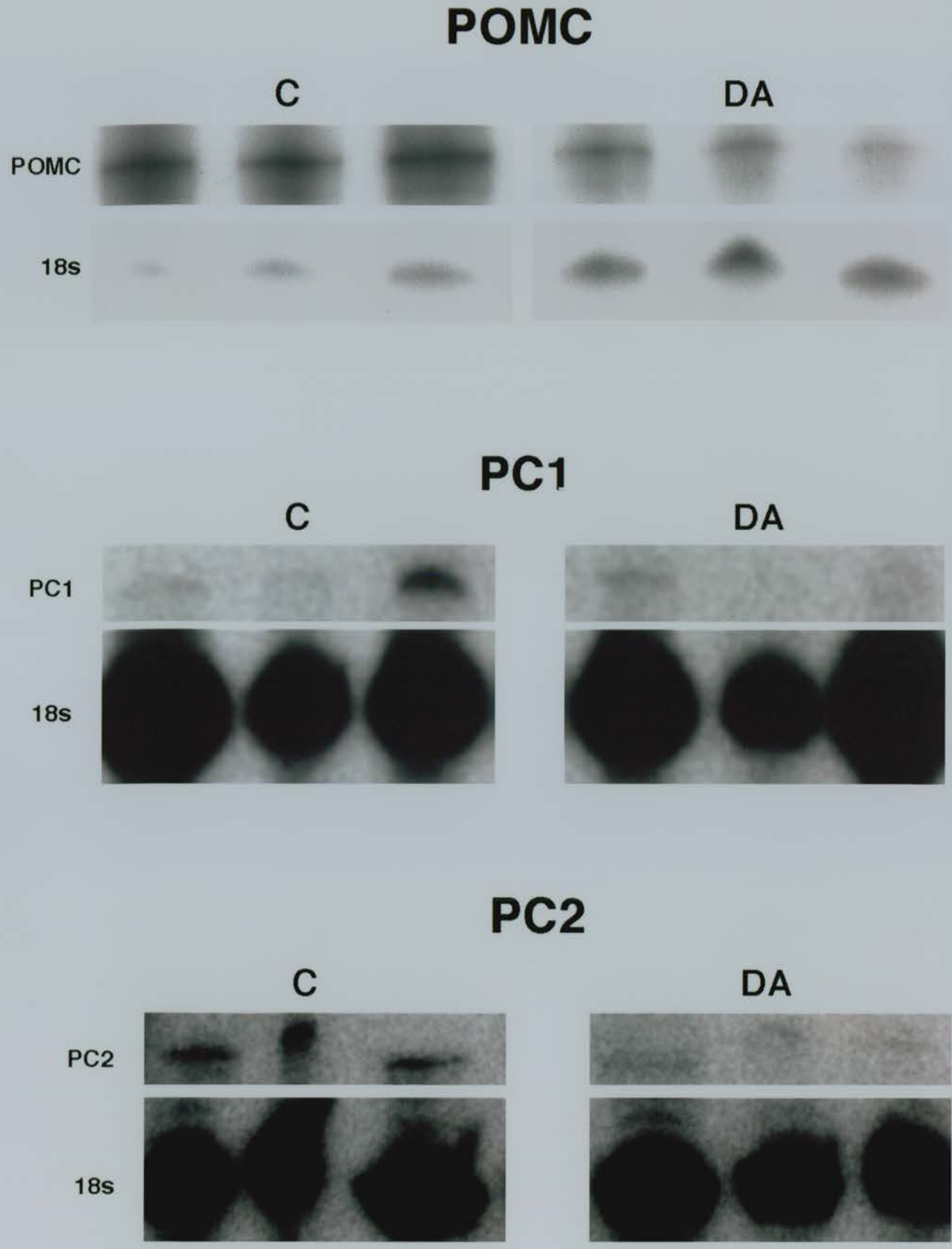
associated with a large decrease in plasma concentrations of αMSH. This is consistent with the general hypothesis that dopamine acts to inhibit the secretion of αMSH and the expression of PC1, PC2 and possibly POMC mRNA. As in the previous study, the changes in POMC mRNA are relatively minor based on the results obtained by in situ hybridisation but marked based on RNase protection assay. The results for the RNase protection assay are equivocal since the procedure protected a mRNA fragment which was smaller than predicted. This may be associated with strong secondary structures occurring where hybridisation conditions were not optimal. Using the results obtained by in situ hybridisation as evidence for the level of POMC mRNA expression, the treatment with dopamine agonist over nearly six weeks induced only a small decrease in POMC mRNA expression. This is consistent with the observations made in the previous chapter where relatively minor changes in POMC gene expression were associated with predicted large changes in circulating αMSH. However, in this case the expression of PC1 and PC2 mRNAs were markedly suppressed implying that the regulation of the processing enzymes is particularly important in the regulation of αMSH secretion. Immunocytochemistry failed to demostrate clear changes in stored peptide levels for αMSH, PC1 and PC2 in the pars intermedia under this treatment, which further shows the limitation in this technique in detecting changes in secretion and peptide activity. Studies in the rat pars intermedia have demonstrated that dopamine agonists inhibit, and dopamine antagonists stimulate, the expression of mRNAs for POMC, PC1 and PC2, indicating stronger effects on POMC mRNA expression than those observed in the current study (Oyarce *et al.*, 1996).

Besides the effects of dopamine agonist on the POMC system, there was a clear, signifiant decrease in cell volume by up to 50 percent in the pars intermedia. This compares with the reverse effect of short term HPD which induces hypertrophy of the melanotrophs (Clarke *et al.*, 1986; Clarke *et al.*, 1983). The decrease in cell size after treatment with dopamine agonist was associated with a trend towards reduced cell proliferation which was just short of significance. This provides some support for the view that where dopamine levels are low, the pars intermedia increases in size due to both hypertrophy and hyperplasia and that these effects are reversed by dopamine which also suppresses αMSH secretion.

In summary, while the animal numbers used where very low, this chapter provides clear evidence that dopamine plays a prominent role in the regulation of αMSH secretion through the inhibitory control of PC1, PC2 and possibly POMC mRNA expression in the Soay ram. The inference is that in autumn, hypothalamic dopamine activity is reduced which permits the activiation of POMC peptide secretion. This seasonal effect is readily reversed by the administration of exogenous dopamine. Thus, it is likely that photoperiod acts, at least in part,

through dopamine to regulate the seasonal cycle in αMSH secretion in the Soay ram.

Figure 6.1 RNase protection assays for POMC, PC1 and PC2 mRNA in the pars intermedia of chronic parlodel treated (dopamine agonist; DA) and untreated control (C) Soay rams. Protected cRNA probe/mRNA band (top) and 18s (internal control) band (bottom). Exposed for 2 days (POMC) or 2 weeks (PC1 and PC2)


POMC
C
DA
POMC
18s
PC1
C
DA
PC1
18s
PC2
C
DA
PC2
18s

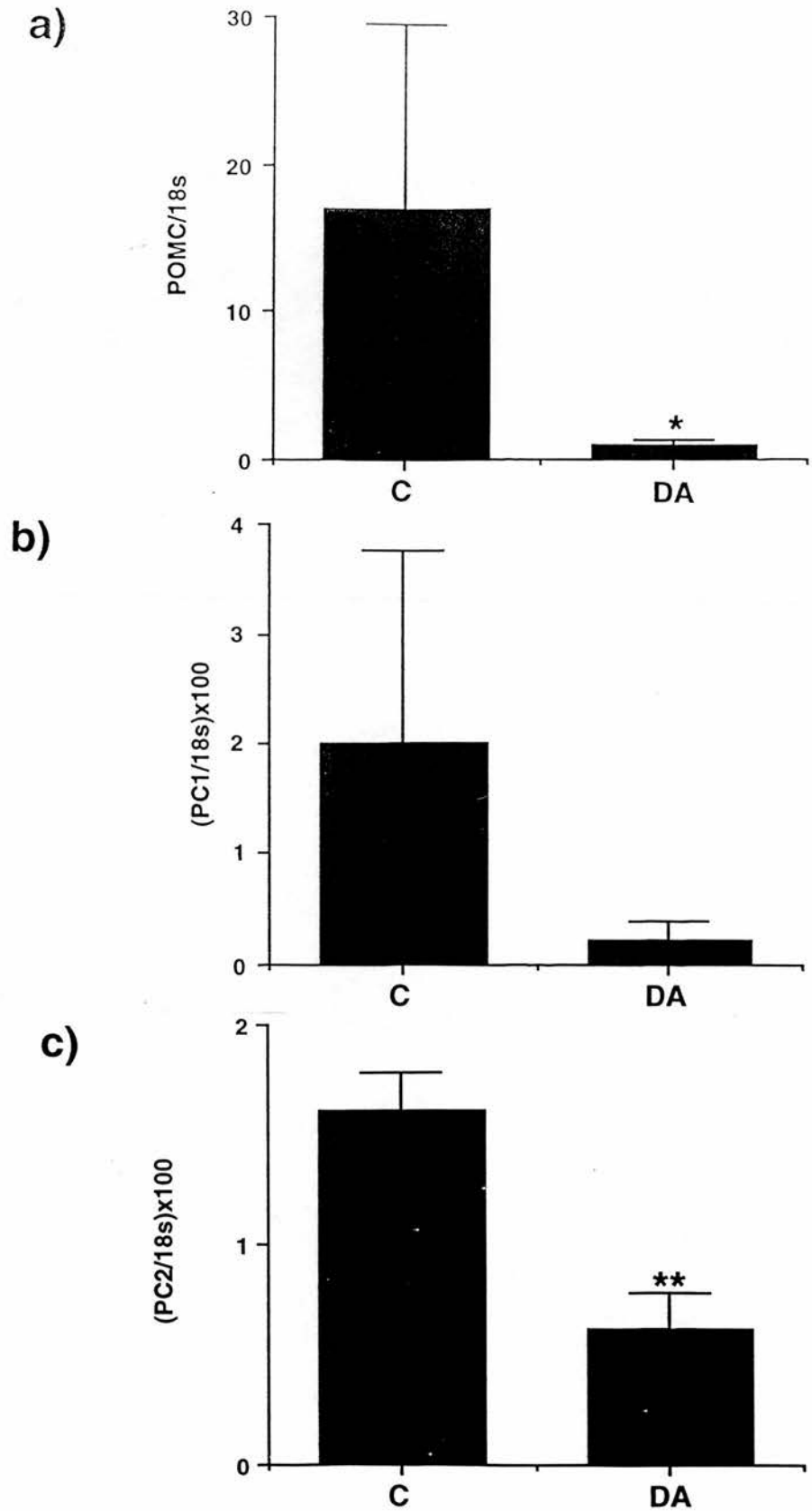
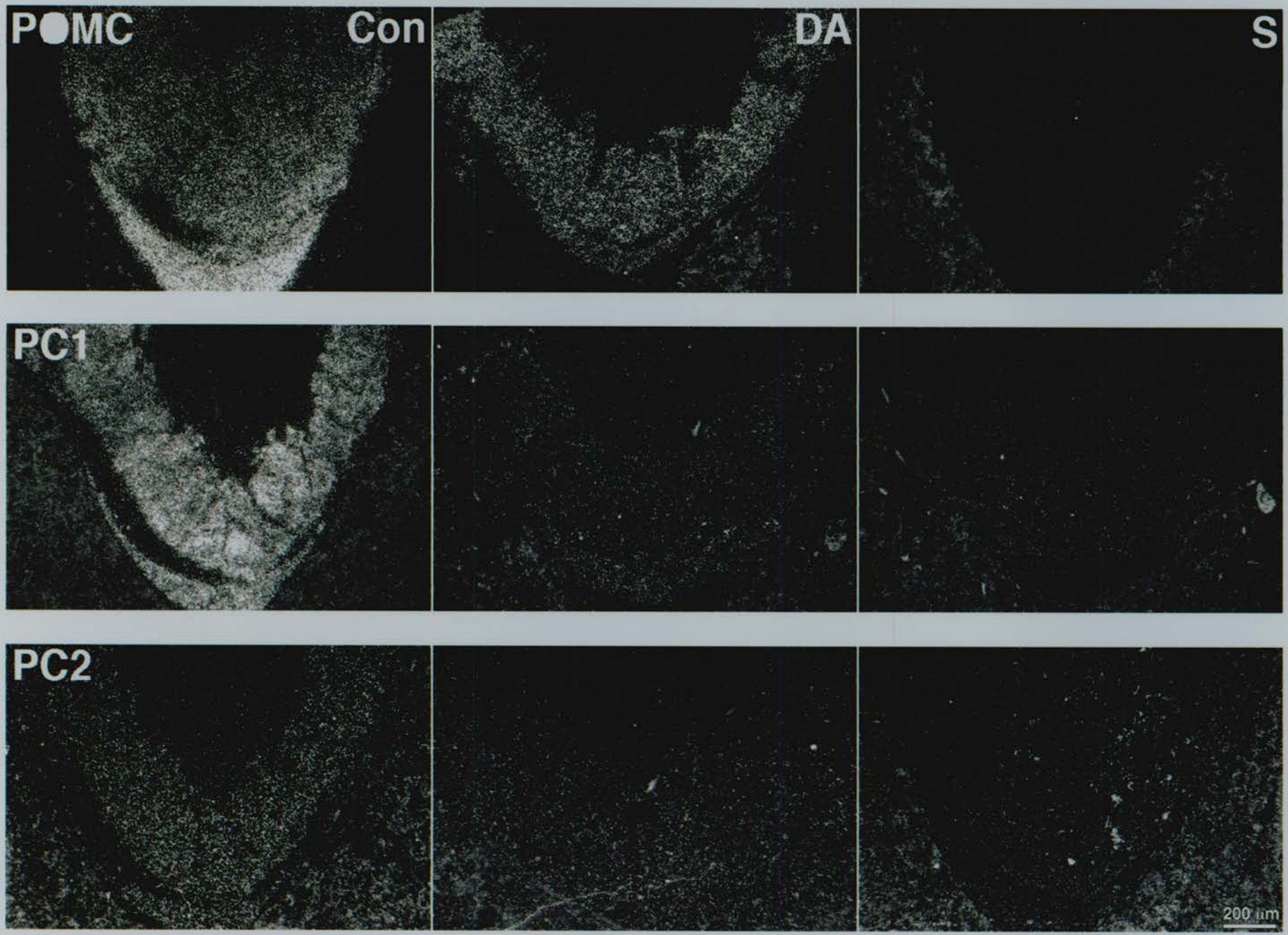


Figure 6.2 Quantitative analysis (intensity of protected fragment divided by intensity of 18s internal control) of POMC (a), PC1 (b) and PC2 (c) RNase protection assays in the pars intermedia of chronic parlodel treated (dopamine agonist; DA) and untreated control (C) Soay rams. POMC and PC2 mRNA expression were both reduced in the pars intermedia of DA-treated compared to control animals ($p<0.05$ and $p<0.01$ respectively, unpaired t-test, transformed data). PC1 mRNA expression also tended to be lower in DA-treated animals but did not reach statistical significance.

Figure 6.3 Representative sections of the pars intermedia of chronic parlodel treated (dopamine agonist; DA) and untreated control (C) Soay rams subjected to in situ hybridisation with POMC (top), PC1 (middle) and PC2 (bottom) antisense and sense control (S) cRNA probes. Sections were exposed for two days (POMC) or six weeks (PC1 and PC2).


POMC
Con
DA
S
PC1
PC2
200 μm

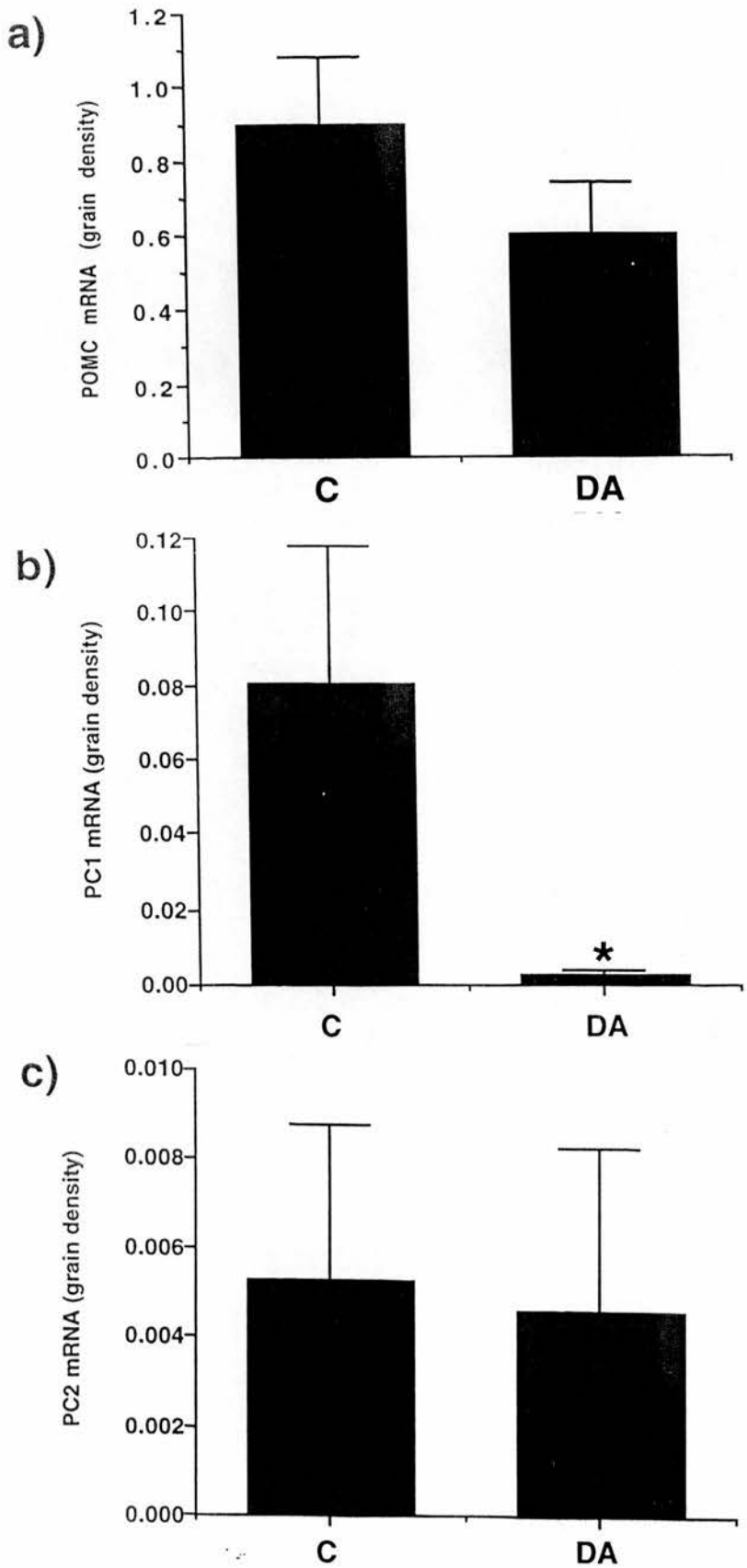
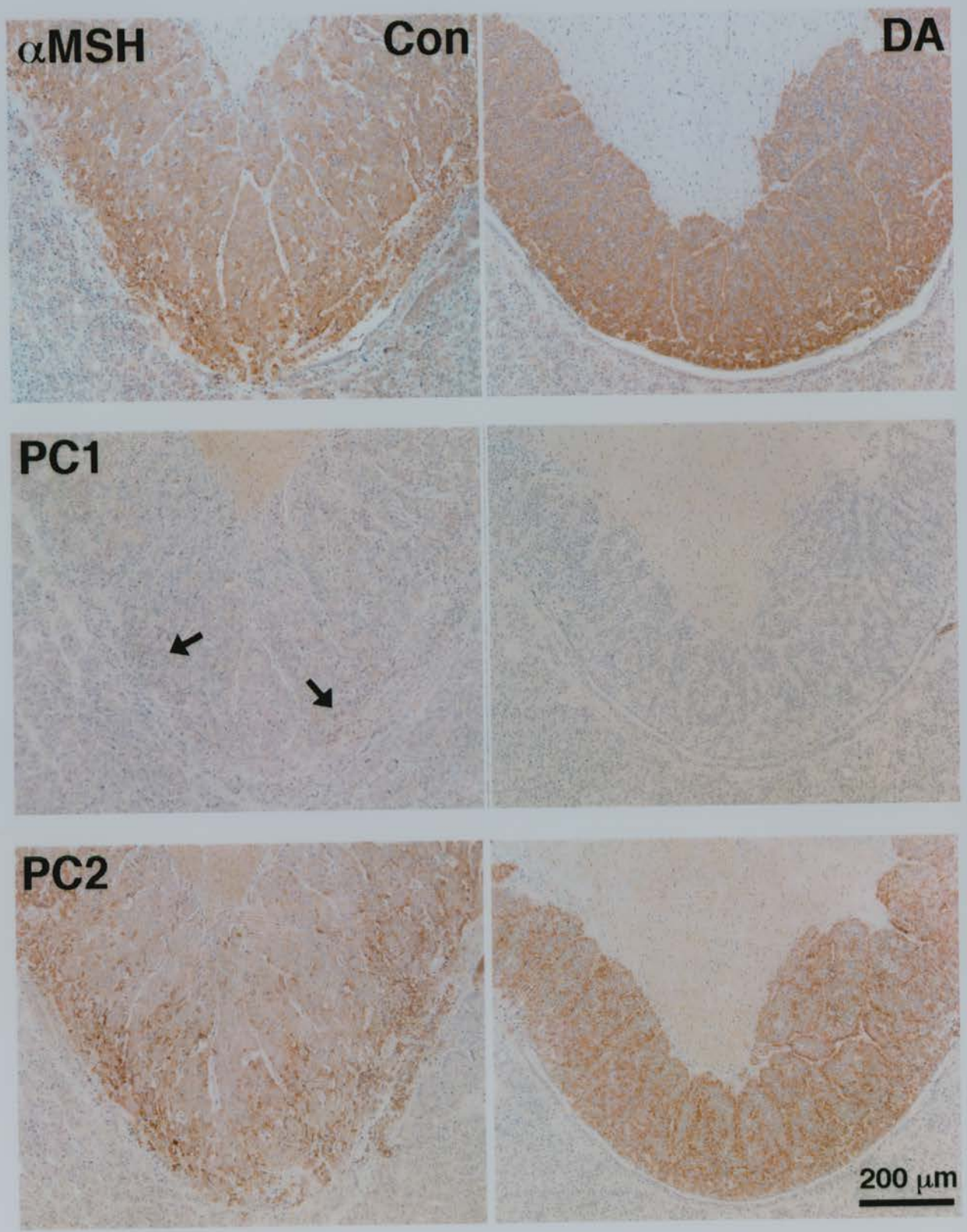


Figure 6.4 Semi-quantitative analysis of POMC (a), PC1 (b) and PC2 (c) in situ hybridisation in the pars intermedia of chronic parlodel treated (dopamine agonist; DA) and untreated control (C) Soay rams. PC1 mRNA expression was significantly reduced in DA-treated compared to control ($p<0.05$, unpaired t-test, transformed data) No significant effect of photoperiod on either POMC or PC2 mRNA expression was detected.

Figure 6.5 Representative examples of αMSH, PC1 and PC2 immunocytochemical staining in the pars intermedia and pars nervosa of chronic parlodel treated (dopamine agonist; DA) and untreated control (C) Soay rams. Arrows indicate areas of more intense PC1 staining. αMSH, PC1 and PC2 staining intensity in the pars intermedia were not significantly affected by treatment.


αMSH
Con
DA
PC1
PC2
200 μm

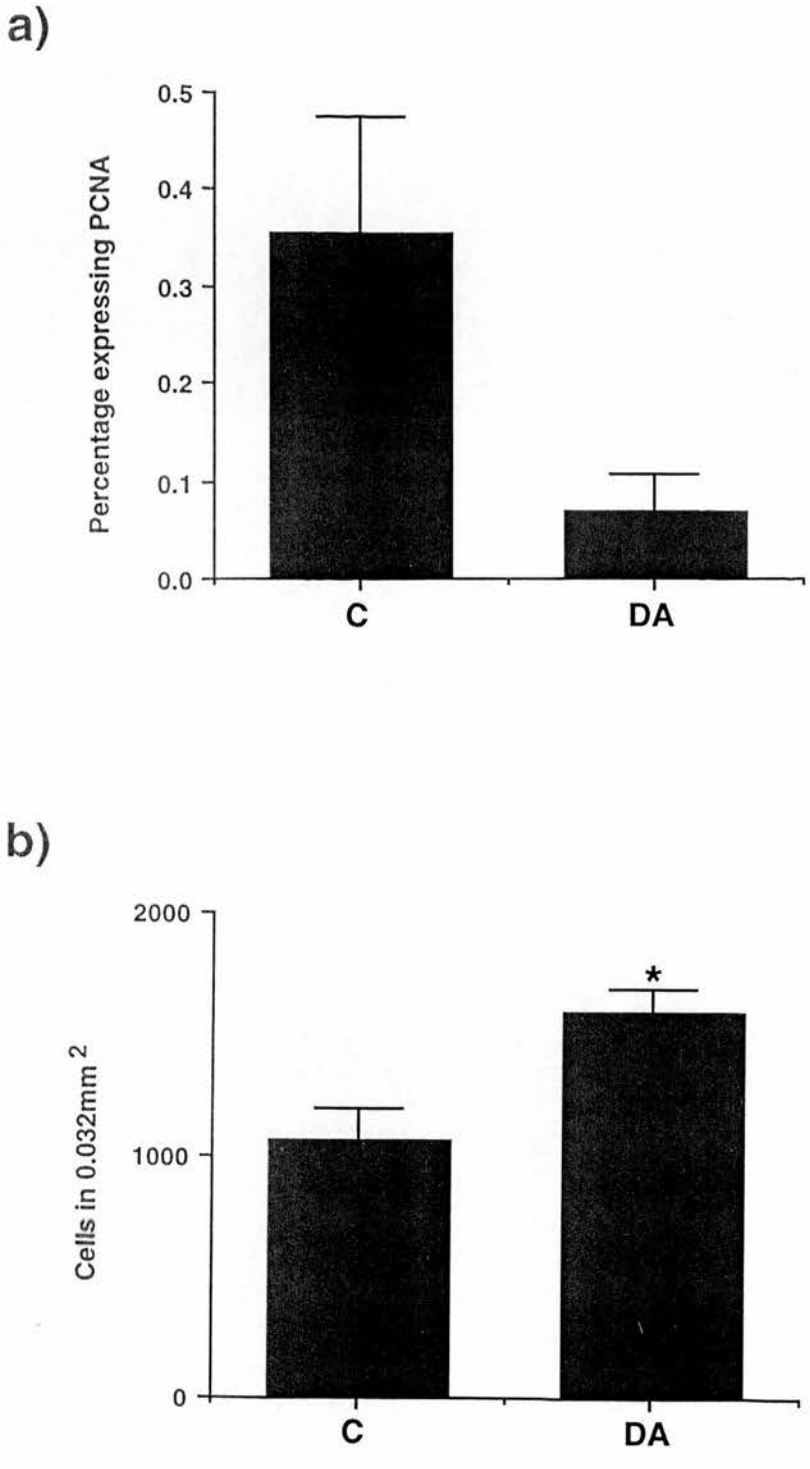


Figure 6.6 Histological analysis of melanotrophs of the pars intermedia in chronic parlodel-treated (dopamine agonist; DA) and control (C) Soay rams. a) PCNA expression tended to be decreased in DA animals but no significant effect of DA was detected. b) Melanotroph cell density was increased by treatment with parlodel suggesting cellular hypotrophy.

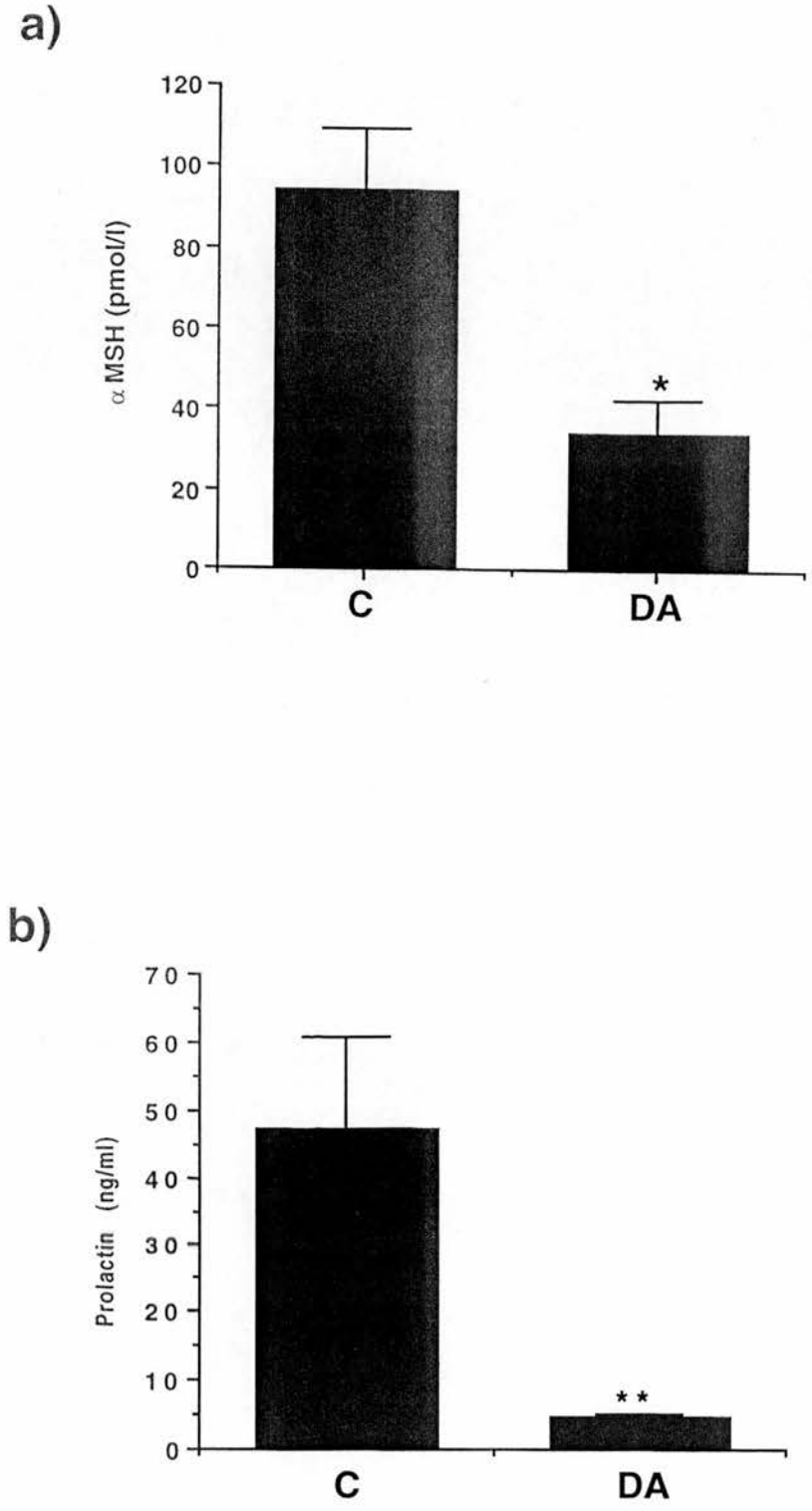


Figure 6.7 Mean plasma hormone concentrations at time of culling (animals killed in August where maximal circulating αMSH concentrations were predicted) in chronic parlodel-treated (dopamine agonist; DA) and untreated control (C) Soay rams. Parlodel reduced circulating levels of both αMSH (a; $p<0.05$, unpaired t-test) and prolactin (b; $p<0.01$).

Chapter 7. General Discussion

In the highly seasonally responsive Soay ram a conspicuous seasonal cycle in circulating αMSH and βEND occurs in which circulating concentrations are maximal in autumn (Ebling and Lincoln, 1987; Lincoln, 1991). Most circulating POMC-derived peptides are derived principally from the pituitary gland (Autelitano *et al.*, 1989; Lundblad and Roberts, 1988; Smith and Funder, 1988). The first aim of this thesis was to confirm that most of the αMSH in circulation in the Soay sheep is secreted by the pars intermedia. Immunocytochemistry revealed that all the cells in the pars intermedia were strongly immunoreactive for αMSH whereas only minimal staining associated with the cortictroph was detected in the pars distalis. Furthermore, both PC2 peptide and mRNA were detected in the pars intermedia but not the pars distalis. Since PC2 is associated with the endoproteolytic processing of POMC to αMSH, these observations indicate that most of the αMSH in circulation is secreted by the pars intermedia as predicted in other breeds of sheep and other species (Engler *et al.*, 1989). Moreover, the general changes in morphology and POMC expression in the pars intermedia seasonally and after HPD and dopamine administration (see below) are associated with predicted and observed changes respectively in circulating αMSH concentrations. A similar distribution of expression of PC1 and PC2 mRNAs and immunoreactivities have recently been reported in the fetal ovine pituitary gland (Bell *et al.*, 1998). Having established that the pars intermedia is the principle source of circulating αMSH in the Soay sheep, this thesis then aimed to study the molecular events in the pars intermedia which underpin the seasonal cycle in circulating concentrations of αMSH and to identify the environmental cues which entrain this cycle.

This thesis has provided evidence that the seasonal cycle in circulating αMSH is generated by seasonal changes in the activity of the pars intermedia. Such seasonal changes in activity were characterised by the seasonal regulation of the expression of the mRNA for the αMSH precursor molecule, POMC. Furthermore, the mRNA expression of the prohormone convertases, PC1 and possibly PC2, which endoproteolytically cleave the αMSH peptide from the POMC precursor, are also regulated seasonally in a similar way to POMC mRNA expression. These changes in mRNA expression are likely to be attributable to changes in gene expression but may also be due to changes in RNA stability since the techniques used do not directly measure the rate of gene transcription. Therefore, the seasonal cycle in circulating αMSH in the Soay sheep is characterised by coordinate seasonal changes in the level of mRNA expression of POMC and its processing enzymes

PC1 and PC2. Moreover, pars intermedia cellular proliferation and cellular volume are also profoundly affected by season and where αMSH-synthetic and secretory activity are maximal, proliferation and cytoplasmic volume are also increased. The failure to detect changes in αMSH immunoreactivity in the tissue may be a limitation of the technique. It is also probable that stored peptide concentrations do not accurately reflect peptide secretion. This is emphasised by studies in the Siberian hamster in which stored αMSH in the pituitary gland is depleted where αMSH secretion into the circulation is increased (Logan and Weatherhead, 1980).

In the natural state this cycle in circulating αMSH is entrained by environmental factors. Photoperiod varies greatly and predictably in latitudes more than 30° from the equator and as such is the most reliable and widely used indicator of season. A role for photoperiod as the principle environmental cue underlying the effect of season on the activity of the pars intermedia is substantiated by the observation that artificial photoperiod can drive a cycle in circulating αMSH in Soay rams housed indoors in which circulating αMSH is maximal under short days (Lincoln and Baker, 1995). This thesis provides some evidence that this artificial cycle is characterised by coordinate changes in the mRNA expression of POMC and both PC1 and PC2, although a statistically significant effect of photoperiod was only detected for PC1 mRNA. An effect of photoperiod on the proliferative activity of the pars intermedia was not detectable. The effect of photoperiod on the pars intermedia was the least robust effect of the experimental regimes. The measurements of cell proliferation and POMC, PC1 and PC2 mRNA expression were only made at two time points which may not have coincided with the maximum effects of photoperiod. The effect of photoperiod on circulating αMSH concentrations is characterised by the rapid development of refractoriness (Lincoln and Baker, 1995) which makes the timing of such measurements critical. Short days are only found to be stimulatory to circulating concentrations of αMSH for approximately eight weeks and similarly long days only exert a powerful inhibitory effect on circulating αMSH for a similar duration (Lincoln and Baker, 1995). However, the results presented in this thesis are consistent with a role for photoperiod as the driving environmental factor behind the seasonal changes in the expression of POMC and its processing enzymes. The observation that seasonally, αMSH concentrations are diminished from autumn to winter further demonstrates that pars intermedia quickly becomes refractory to the effect of short days (Lincoln and Baker, 1995).

This thesis illustates that the effect of photoperiod on the activity of the pars intermedia is mediated through the hypothalamus. This is based on the absence of an effect of photoperiod on the expression of POMC, PC1 and PC2 mRNAs or cellular proliferation in the pars intermedia of HPD animals. These results are

consistent with previous studies which demonstrate that the removal of direct hypothalamic innervation of the pars intermedia by HPD abolishes the ability of photoperiod to entrain the cycle in circulating αMSH (Lincoln and Richardson, 1998). Furthermore, no effect of photoperiod on αMSH immunocytochemical staining in the pars intermedia was detected in HPD animals although increased αMSH immunoreactivity was detected in the corticotroph in all HPD, compared to intact animals. This effect of HPD may be due to increased processing of POMC-derived peptides resulting from the longer term storage of POMC-derived peptides in the corticotroph associated with the removal of stimulatory hypothalamic influences. It is possible that basal secretion of such αMSH immunoreactive peptides from the corticotroph contributes to the increased circulating concentrations of αMSH observed in the HPD animal (Lincoln and Richardson, 1998). However, this increase in circulating αMSH after HPD is likely to be principally due to the increased activity of the pars intermedia and suggests that the regulation of the pars intermedia by the hypothalamus is principally inhibitory. In the longer term, however, circulating αMSH is reported to decline in the HPD animal (Lincoln and Richardson, 1998). The results presented in this thesis show a general long term decline in the expression of mRNAs for POMC, PC1 and PC2 compared to intact SD animals, although this was only statistically significant for PC1 mRNA. This long term decline in activity suggests that the pars intermedia requires hypothalamic influence for normal function in the long term.

In spite of the low number of animals studied, this thesis has presented evidence that a large component of the inhibitory influence which is exerted on the pars intermedia by the hypothalamus is dopaminergic. This is based on observation that the administration of dopamine agonist to Soay sheep at the peak of the αMSH cycle resulted in marked decline not only in circulating concentrations of αMSH, but also in the mRNA expression of PC1 and PC2. Dopamine agonist also had an impact on pars intermedia cellular morphology (reduced cytoplasmic volume) and proliferation (reduced). Given the low sample size used in this study, the effect of dopamine on the pars intermedia was the most robust experimental regime observed in this thesis. The effect of dopamine on the ovine pars intermedia is similar to that reported in the rat where dopamine agonists are inhibitory to both the mRNA expression of POMC, PC1 and PC2 (Oyarce *et al.*, 1996) and the cellular proliferative activity in the pars intermedia (Chronwall *et al.*, 1987). The large decrease in circulating αMSH following treatment with dopamine agonist was not associated with any changes in pars intermedia αMSH immunoreactivity. This further indicates that immunocytochemistry for αMSH is a poor indicator of secretory activity in the pars intermedia.

The only other well studied example of a long term regulation of the activity of the pars intermedia in response to changes in the external environment is that observed in the amphibia, where αMSH has a role in the regulation of skin colour in background adaptation. The ability to respond to changing, visually perceived environmental conditions by alterations in skin colour is achieved through long term changes in pars intermedia activity and circulating concentrations of αMSH (Tonosaki *et al.*, 1995). The amphibian pars intermedia is innervated by dopaminergic neurons originating in the hypothalamus (Tonosaki *et al.*, 1995), as in the sheep (Gayrard *et al.*, 1995). Also as in the sheep, isolation of the amphibian pars intermedia from the hypothalamus results in hypersecretion of αMSH as indicated by inappropriate black pigmentation and the failure to respond to changes in background colour (Etkin, 1935). The changes in activity of the pars intermedia under such changes in environmental lighting conditions are, as in the Soay sheep, characterised by changes in the expression of the genes for POMC and its processing enzymes with a 20 to 30 fold increase in POMC mRNA expression in frogs adapted to black background conditions (Ayoubi *et al.*, 1992). Not only POMC, but PC2 and 7B2, a molecular chaperone associated with PC2 maturation, are coordinately regulated with POMC in the amphibian pars intermedia in this model (Braks *et al.*, 1992). Furthermore, the degree of acetylation of αMSH is also altered in a coordinated way by these environmental changes which is associated with an increase in the biological activity of αMSH (Maruthainar *et al.*, 1992). Moreover, hypothalamically-mediated adaptation to background elicits not only changes in gene expression, but also changes in POMC RNA stability (Ayoubi *et al.*, 1992). Taken together these observations suggest that the regulation of circulating POMC-derived peptides occurring in the amphibian is regulated at the levels of gene transcription, RNA degradation and post-transcriptional modification.

The data presented here allows us to construct a hypothetical model of the events which generate the seasonal cycle in the activity of the pars intermedia (Figure 7.1). In this model it is proposed that the effects of season are mediated primarily due to the annual cycle in photoperiod. The effects of photoperiod are mediated through changes in melatonin secretion which generates a humoral signal that encodes photoperiod and acts at specific sites in the hypothalamus to influence the activity of dopaminergic neuronal systems that innervate the pars intermedia. Light transmitted from the retina by the retino-hypothalamic tract, a non visual neural pathway to the hypothalamus. The light has two effects. The first is that it acts to entrain the endogenous circadian rhythm generating system in the suprachiasmatic nucleus which governs daily cyclicity inducing the rhythmic secretion of melatonin. Secondly, light acts via the sympathetic innervation of the

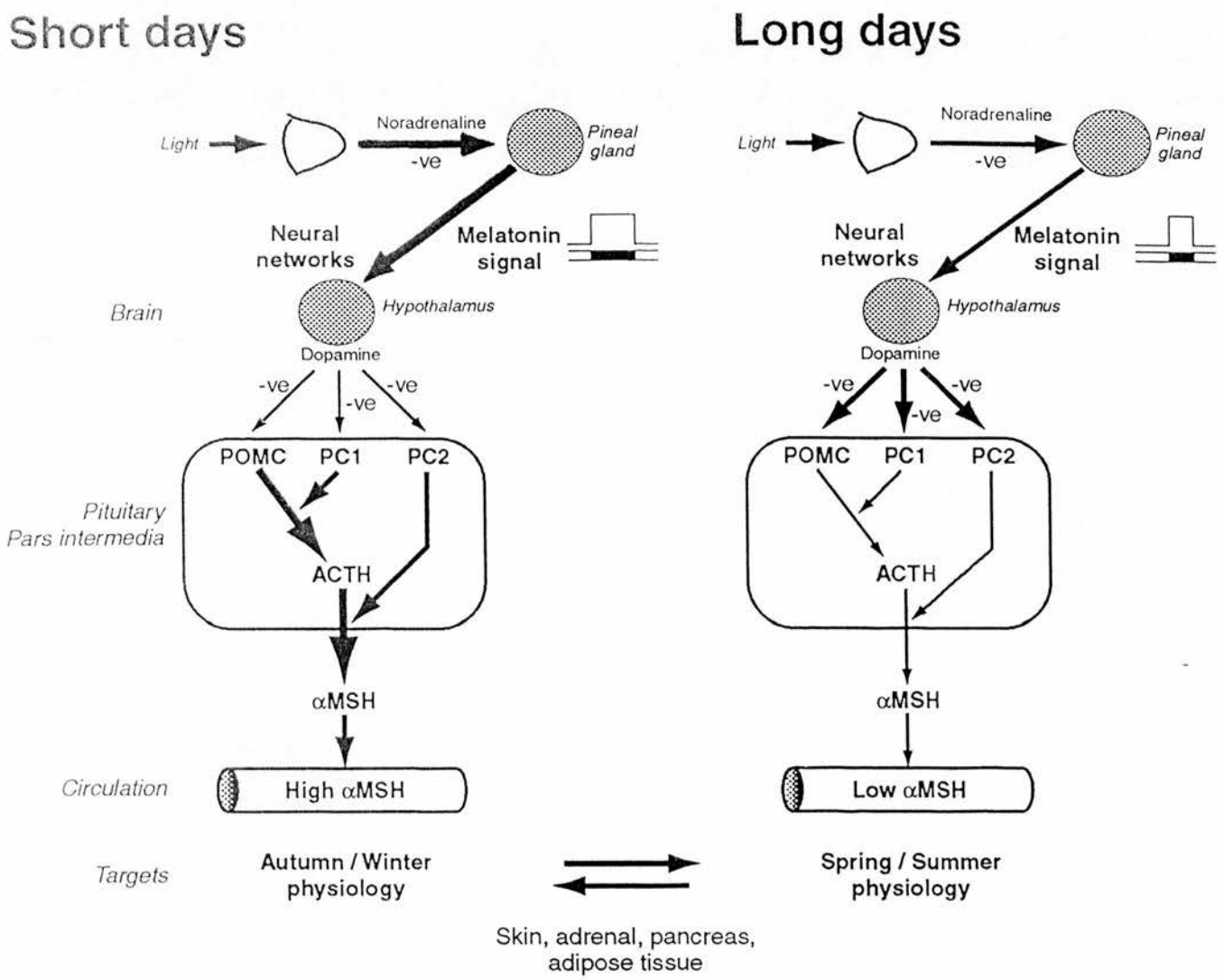


Figure 7.1 Schematic model showing the proposed regulation of the activity of the pars intermedia by photoperiod.

pineal gland to inhibit melatonin secretion, the consequence of which is that melatonin is secreted only at night and so the duration of melatonin secretion reflects the length of the dark phase. The evidence that melatonin mediates the effects of photoperiod is based on the observation that the administration of continuous melatonin (through subcutaneous implants) to Soay sheep exposed to long days results in increased circulating αMSH, so mimicking to effects of short days (Lincoln, 1991). More specifically, the administration of microimplants of melatonin directly to the medial-basal hypothalamus activates the activity of the pars intermedia (as judged by the measurement of circulating βEND), demonstrating that the medial-basal hypothalamus is the site of action of melatonin in influencing the photoperiodic relay system to the hypothalamus (Lincoln and Maeda, 1992a). Under short days, the long duration melatonin signal results in inhibition of dopamine activity and disinhibits the function of the pars intermedia resulting in increased αMSH and βEND secretion. The photoperiodic regulation of the pars intermedia is associated with the coordinated activiation of POMC mRNA and its associated endoproteolytic enzymes which are involved in the production of the fully mature hormone. Conversely, under long days the short duration melatonin signal acts in the hypothalamus to promote dopamine secretion resulting in the inhibition of the complex internal cellular machinery responsible for generating the mature αMSH peptide. In this model, the control of mRNA expression and post-translational processing enzymes contribute to the production of αMSH. It is also probable, as has been demonstrated in the frog, that the processing of the POMC molecule to αMSH is controlled further by the regulation of acetylation as well as exoproteolytic processing and α-amidation.

Seasonal cycles in αMSH and βEND is unlikely to be restricted to the Soay sheep since the pituitary content of αMSH also varies seasonally in the Siberian hamster (Logan and Weatherhead, 1980). Similar cycles are likely to occur in other sheep breeds but may be attenuated in domestic breeds as a result of selective breeding. The Soay sheep is a feral breed and like wild sheep the Soay displays particularly robust seasonally-regulated changes in pelage and food intake as well as reproduction (Argo and Smith, 1983; Lincoln, 1991). The seasonal changes in these physiological parameters are characterised by seasonal cycles in circulating levels of other pituitary hormones such as prolactin and FSH and represent important adaptations for survival in a predictably seasonally-changing environment.

With respect to the functional significance of the αMSH system, there is an extensive literature indicating that αMSH and βEND have a role in the regulation of appetite and body weight (Davis *et al.*, 1983; Deutch and Martin, 1983; Genazzani *et al.*, 1986; Huszar *et al.*, 1997; Margules *et al.*, 1978; Mountjoy and Wong,

1997). Most of this literature describes the effects of central rather than circulating αMSH, which may not be able to pass through the blood-brain barrier. While αMSH is known to have a role in the regulation of appetite in the hypothalamus (Huszar *et al.*, 1997; Mountjoy and Wong, 1997), the physiological functions of peripheral αMSH in the sheep are poorly understood. However, the seasonal cycles of αMSH and βEND correlate closely with voluntary food intake and body weight in the Soay sheep (Ebling and Lincoln, 1987; Lincoln and Baker, 1995; Lincoln and Richardson, 1998), which are maximal in late summer (July, August and September) and fall to a minimum in winter and spring (December to April). In contrast to the localised effects of αMSH in the hypothalamus, the secretion of αMSH and βEND from the pars intermedia represents a conspicuous signal to many peripheral tissues which possess receptors for these peptides. A role for αMSH in the regulation of carbohydrate metabolism is also implicated by the distribution of these receptors. While it is not clear whether the βEND secreted by the pars intermedia is bioactive, five subtypes of αMSH receptors have been described, two of which are expressed in adipose tissue (Mountjoy and Wong, 1997). One of these is the type one melanocortin receptor (MC1-R) which occurs in the pigment cells of the skin and is thought to mediate the effects of αMSH in the regulation of pigmentation. However, this receptor is also expressed in adipose tissue and in macrophages (Mountjoy and Wong, 1997). The second receptor type (MC2-R) is principally an ACTH receptor, but also has a low affinity for αMSH. MC2-R mediates the stimulatory effects of ACTH on the adrenal cortex but is also expressed in adipose tissue and the adrenal gland (Mountjoy and Wong, 1997). The third receptor subtype (MC3-R) is expressed centrally and in the placenta, duodenum, the pancreas and the stomach while the type four receptor (MC4-R) is expressed centrally in the brain, spinal cord, sympathetic nervous system and muscle (Mountjoy and Wong, 1997). MC4-R is implicated in the central control of appetite since its inactivation in the mouse results in hyperphagia and obesity (Huszar *et al.*, 1997). The fifth receptor (MC5-R) has a more widespread pattern of peripheral expression than any of the other melanocortin receptor subtypes and is particularly highly expressed in exocrine tissues (van der Kraan *et al.*, 1998).

Many ungulates show robust seasonal cycles in voluntary food intake and body weight particularly in semi domesticated and wild animals such as the Soay sheep and the red deer (Ebling and Lincoln, 1987; Loudon and Jabbour, 1994). This seasonally modified behaviour, which is characterised by fat storage and hyperphagia while food is abundant, has evolved to anticipate and exploit seasonal changes in environment and optimise physiology to cope with the deprivations of winter. This type of adaptation to seasonal food availability is taken to extremes in hibernators such as ground squirrels which display conspicuous seasonal cycles in

body weight characterised by hyperphagia and fat deposition in summer and autumn followed by winter anorexia and weight loss (Davis, 1976; Mrosovsky and Sherry, 1980). The cyclical changes in voluntary food intake and metabolism are associated with a wide range of physiological changes including adipose tissue sensitivity to insulin (Castex and Sutter, 1981b; Feist *et al.*, 1986; Florant and Greenwood, 1986) alterations in pancreatic function (Castex *et al.*, 1979; Castex and Sutter, 1981a; Florant *et al.*, 1985) and variations in circulating thyroid hormones which may seasonally elevate metabolic energy expenditure (Jallageas and Assenmacher, 1983; Rickart, 1986; Young, 1984). Such peripheral seasonal alterations in adipose and pancreatic activity are likely to be associated with seasonal cycles in peripheral humoral factors, such as αMSH and βEND.

The results obtained in the course of this study were often subject to a high degree of variability. The comparative difficulty inherent in the maintenance of large animals such as the sheep precludes the use of large numbers of animals. Moreover, as well as contributing to high statistical variance, the low sample sizes used in the experiments prevents sampling at many time points. Since the Soay sheep is a feral breed, animals are subject to high genetic polymorphism. and so may behave differently to each other in terms of the magnitude and timing of their response to the experimental regimens imposed on them. Future studies may require larger sample sizes and sampling at more time points to unequivocally establish the effects of season and photoperiod on the activity of the pars intermedia POMC system. The persistence of seasonal changes in the activity in the pars intermedia of the Soay sheep implies a physiological function for the secretory products of this tissue. Future work would therefore focus on the physiological significance of the seasonal cycle in αMSH. To establish the physiological significance of the seasonal cycle in αMSH and its target tissues, receptor studies could be undertaken to map the distribution of the receptor subtypes and invetigate whether the expression of any of these receptor populations are regulated seasonally, as their endocrine ligands are. This may also help to establish whether the normal ligands for these receptors are endocrine rather than paracrine.

This thesis demonstrates that the seasonal cycle in circulating αMSH is associated with changes in pars intermedia activity and morphology in the Soay sheep. This seasonal cycle in the activity of the pars intermedia is characterised by changes in gene expression of POMC, PC1 and PC2 and cellular proliferative activity and is driven by photoperiod, the effect of which is transduced through the inhibitory regulation of the pars intermedia by the hypothalamus. Dopamine is a major component, possibly the principle component of the regulation of the pars intermedia by the hypothalamus which conveys the effect of season to the pars intermedia. The physiological significance of the seasonal cycle in circulating

αMSH concentrations generated by the changes in activity of the pars intermedia described in this thesis is poorly understood, but is likely to be an important mechanism which has evolved to anticipate and optimise survival in an environment in which food availability changes seasonally.

Appendix I: Commonly Used Buffers and Agars

Buffers

1 x SSC
0.15M sodium chloride
0.015M sodium citrate
at pH7.0

1x TBE
0.089M Tris base
0.089M boric acid
10mM EDTA

1 x TAE
0.04M Tris base
0.02M sodium acetate
1mM EDTA
at pH7.2

20 x SSPE buffer
3M $NaCl_2$
200mM NaH_2PO_4
20mM EDTA
at pH 7.4

50 x Denhardts
5g BSA
5g polyvinylpyrrolidine
5g ficoll
in 500ml

TE buffer
10mM Tris-HCl
1mM EDTA
at pH8.0

SM buffer
10mM Tris, pH7.4
10mM $MgSO_4$
0.01% gelatin

1 x STE
100mM NaCl
10mM Tris
1mM EDTA

MOPS Buffer
200mM MOPS
10mM EDTA
50mM Sodium Acetate
at pH 7.0

Agars

Luria Bertani (LB) broth

10g bacto-tryptone
5g bacto-yeast extract
10g NaCl
in 1 litre water

LB-agar

LB-broth
with 1.5% bacto-agar

Tissue fixative

Bouins Reagent

500ml 40% formaldehyde
100ml acetic acid
2 litres saturated picric acid
filter before use.

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